PHOSPHOLIPIDS OF PHOTORECEPTOR MEMBRANES IN RELATION TO THE BINDING SITE OF RETINALDEHYDE IN RHODOPSIN

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CONTENTS

ABBREVIATIONS

Pl-ase C	phospholipase C
Pl-ase A	phospholipase A
Lipid-P	lipid-phosphorous
S.E.	standard error of the mean
vol.	volume
w/w	weight by weight
TRIS	tris-(hydroxymethyl)aminomethane
ATP-ase	adenosinetriphosphatase
min	minute(s)
hr	hour(s)
g	gravity
$R_{C,H,A}$	phospholipase C treated-hexane extracted, phospholipase A treated rod outer segment preparation.
$R_{C,H,A,S}$	phospholipase C treated-hexane extracted, phospholipase A treated, serum albumin extracted rod outer segment preparation.

GENERAL INTRODUCTION

A central problem in the study of the visual mechanism is how a photon absorbed by a visual pigment molecule can lead to a stimulation of the synaptical end of the photoreceptor cell A hypothesis for the mechanism of this process was formulated by Bonting and Bangham in 1967 They suggested that illumination of rhodopsin causes a transisomerisation of retinaldehyde from its original binding site to another aminogroup in the rhodopsin membrane complex This shift of retinaldehyde would cause a sudden increase in cationic permeability of the saccular membrane of the photoreceptorcel, thus causing a photoreceptor current which could activate a cholinergic mechanism at the photoreceptor bipolar synapse A crucial point in this hypothesis is the chromophore binding site before and after illumination

While it is known that retinaldehyde in photolyzed rhodopsin is linked via an aldimine bond to the ϵ-aminogroup of lysine, the binding site in native rhodopsin is still uncertain Recently, evidence has been published for an aldimine link between retinaldehyde and an aminogroup-containing phospholipid in native rhodopsin Conflicting evidence of an indirect nature has also been reported The main purpose of our study has been to determine definitively whether or not an aldimine link between a phospholipid and retinaldehyde exists in native rhodopsin

The strategy followed was to remove the phospholipids as completely as possible without affecting the binding of the chromophore, and then to see whether the molar ratio phospholipid retinaldehyde for the two relevant phospholipids had fallen below 1 Prior to this, the molar absorbance coefficient of rhodopsin was redetermined in view of its importance in determining pigment concentrations and of recent controversy concerning the value of this constant

After a quantitative analysis of the phospholipid pattern of the native rhodopsin preparation, the removal of phospholipids by means of treatment with phospholipase C followed by hexane extraction was studied Additional removal of phospholipids was accomplished by treatment with phospholipase A followed by extraction with serum albumin Analysis then showed that the molar ratio's for both amino group containing phospholipids to retinaldehyde were far less than 1, while the spectral integrity of the rhodopsin was only slightly affected. This definitely rules out the possibility of a phospholipid-

retinaldehyde link in native rhodopsin. In addition, the effect of the phospholipid removal on the regenerating capacity of the rhodopsin was investigated.

CHAPTER 1

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE VISUAL SYSTEM

1.1. MORPHOLOGY

The vertebrate retina is a layer of tissue with a thickness of approx. 0.05 mm which covers the posterior half of the eyecup. Looking from the scleral to the vitreous side of the retina the following layers can be distinguished: the pigment epithelium, the photoreceptor cells, the bipolar cells, the ganglion cells and the nerve fibre layer. Thus the incoming light has to pass through the major part of the retina before it can reach the photoreceptor cells. The photoreceptor cells absorb the incoming light and convert it into an electrical impulse which goes via the bipolar and ganglion through the optical nerve to the visual cortex of the brain.

Two types of photoreceptor cells can be distinguished in the vertebrates: the rods and the cones. They are both elongated cells, aligned roughly in the direction of the incoming light. Rods are dimlight photoreceptor cells and are not colour sensitive, while cones function only in bright light and mediate colour vision.

Morphologically the photoreceptor cells can be divided into an inner segment and an outer segment (Fig. 1). The inner segment contains the nucleus, the mitochondria and endoplasmatic reticulum of the cell and has a synaptical end which is in close contact with the bipolar and horizontal cells of the retina.

The outer segment shows electronmicroscopically a lamellar structure. In the cones these lamellae originate from invagination of the plasma membrane of the photoreceptor cells. Going from the base to the top of the outer segment the diameter of these infoldings decreases giving the outer segment its typical conical shape. In rods the invagination takes place at the base of the outer segment, and then the lamellae appear to be pinched off resulting in the rod sacs. The diameter of these sacs at the base equals that at the top, giving the rod outer segments a cylindrical form. Each rod outer segment

possesses approx. 500 - 2,000 of these regularly piled, flattened sacs, the whole stack being enclosed by the plasma membrane.

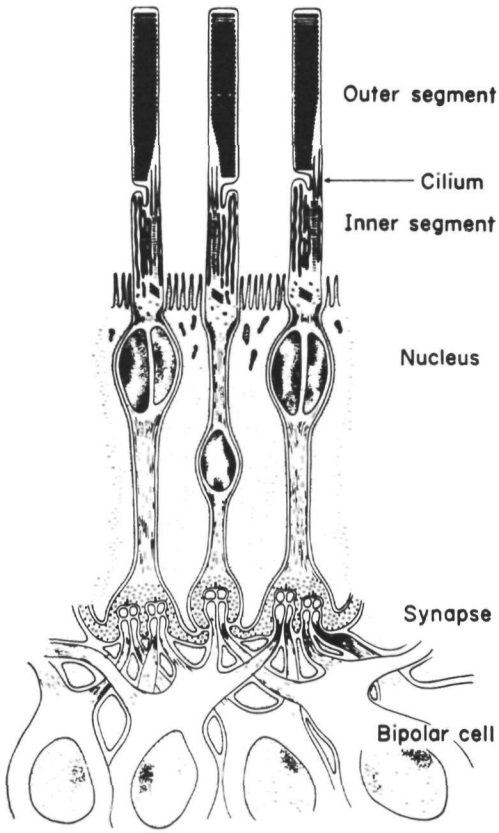


Figure 1. Structure of rod photoreceptor cell. After Sjöstrand (1961).

The outer segment contains no mitochondria and ribosomes which suggests that they depend for energy metabolism and protein synthesis on the inner segment. It has been shown by Young and Droz (1968) that upon

injection of radioactive amino acids in vivo, the first labelling takes place at the site of protein synthesis in the inner segment. In the next stage the label moves upward through the cilium to the base of the outer segment, where the newly synthesized protein is used for a continuous renewal of rod sacs. The upper half of the outer segment is in close contact with the pigment epithelia cells. This close contiguity between the outer segments of the photoreceptor cells and the pigment epithelium may serve multiple purposes. The autoradiographic studies of Young and Droz (1968) indicate that at the top of the outer segments rod sacs are removed by lysosomal activity of the pigment epithelium. In addition, there is evidence that retinaldehyde on being liberated from the visual pigment after intensive illumination moves to the pigment epithelium in the form of free retinol or its fatty acid ester (Dowling, 1960; Andrews and Futterman, 1964). Thence it would be made available for the regeneration of the visual pigment. It is not clear at the moment, whether this is the case for the small amounts of retinaldehyde liberated during low level physiological illumination, or only for the excess retinaldehyde liberated during brief, saturating illumination occurring only under experimental conditions.

The inner and outer segments are connected by the cilium, a very narrow stalk visible only with the electron microscope. The cilium plays a role in the transport from the inner segment to the outer segment of substances necessary for the formation, the energy supply and the functioning and the rod sacs, and possibly in the conduction of the visual excitation impulse over the large distance between the outer segment and the synaps. The anatomy of the other constituents of the visual system will not be discussed. For more extensive reviews of the morphology of the visual system the reader is referred to the papers of Fernandez-Moran (1962), Moody and Robertson (1960) and Sjöstrand (1961, 1968).

1.2. THE VISUAL PIGMENT RHODOPSIN

1.2.1. *Absorption spectrum*

The visual pigment rhodopsin occurring in vertebrate rods is part of the lipoprotein complex constituting the rod sac membrane and is insoluble in

water. The light absorption spectrum of rhodopsin is an important tool in the determination both of its properties and its concentration. Although spectral measurements can be obtained from aqueous suspensions of visual cells it is simpler to work with extracts prepared with detergents (Dartnall, 1961). The earliest investigators used bile salts to solubilize the visual pigment from the rod cells. In 1931 Tansley introduced the use of an aqueous solution of the colourless plant glycoside digitonin. This natural detergent is still used very frequently for the extraction and spectral measurement of visual pigments. Other detergents used nowadays are Triton X-100, sodium deoxycholate, cetyltrimethyl ammonium bromide (CTAB) and emulphogene. The spectrum of rhodopsin in aqueous detergent solution has been shown to be identical to that obtained from rhodopsin in intact visual cells (Dartnall, 1961).

The absorption spectrum of rhodopsin (Fig. 2) shows three peaks. One of the major peaks, the α-peak, lies at wavelengths varying from 430 to 560

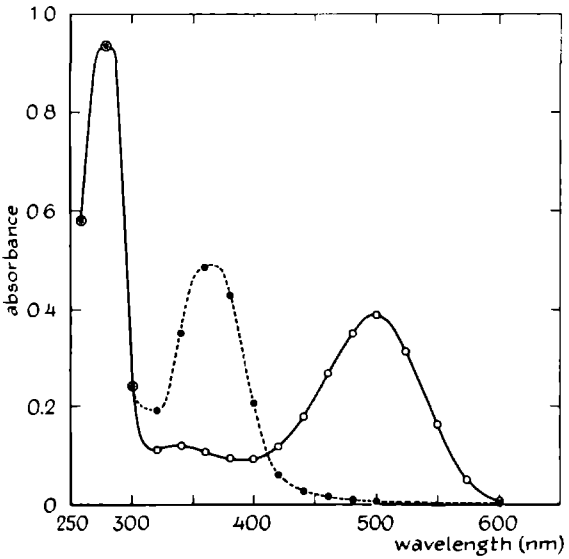


Figure 2. Absorption spectra of cattle rhodopsin in 1%/o digitonin, before (solid line) and after (dashed line) illumination in the presence of hydroxylamine.

nm dependent on the animal species from which the rhodopsin is derived. The other major peak, the γ-peak, has its maximum at 278 nm and originates from the protein present in the visual pigment complex. The third and minor peak, the β-peak, is observed between 340 and 370 nm.

Illumination causes a drastic change of the absorption spectrum of rhodopsin (dashed line in Fig. 2). The α-peak, which for cattle rhodopsin is located at 500 nm, disappears and a new maximum at 380 nm appears, which covers the small β-peak. The γ-peak does not alter upon illumination. Disappearance of the α-peak takes place also when rhodopsin is exposed to protein-denaturing conditions such as heat, strongly acid or alkaline pH-values and polar organic solvents. The height of the α-peak can therefore be regarded as a criterion for the integrity of the rhodopsin complex and as a measure of its concentration.

1.2.2. *Photolysis*

Illumination of rhodopsin results in a change of colour from reddish-purple to yellow. This event has therefore been called "bleaching". Since the process has been shown to result eventually in a splitting off of the chromophoric retinaldehyde group, it has also been named "photolysis". The changes taking place in the rhodopsin molecule during photolysis have been studied in detail by spectrophotometry at low temperature (Fig. 3). The low temperatures were required to reduce the very fast reaction rates sufficiently to permit the spectral measurements.

Illumination of rhodopsin in aqueous glycerol at -196^{o} resulted in a shift of the absorption maximum from 500 nm to 543 nm (Yoshizawa et al, 1960; Yoshizawa and Wald, 1963). The product thus formed has been called prelumirhodopsin and is the first photo-product that can be obtained from rhodopsin. The only change that occurs at this low temperature is the photoisomerisation of 11-cis retinaldehyde to the all-trans isomer (Yoshizawa and Wald, 1963). This change is the only step in the photolytic process that requires light. All further steps are, therefore, thermal reactions. On warming of prelumirhodopsin in the dark to a temperature of -140^{o}C lumirhodopsin is formed which absorbs maximally at 497 nm (Hubbard and Kropf, 1959 b). Further raising of the temperature to -40^{o}C results in the formation of meta-

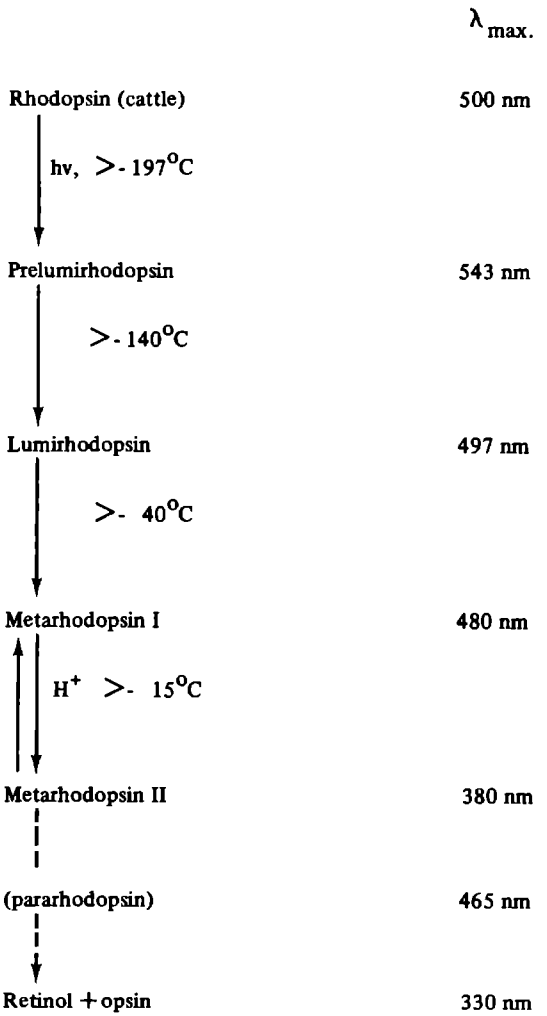


Figure 3. Scheme of the photolytic process of vertebrate rhodopsin.

rhodopsin I (λ max. = 480 nm), (Wald et al, 1950). Above -15°C this product is reversibly transformed into metarhodopsin II, (Matthews et al, 1963). This

step is characterized by the largest spectral shift (100 nm). The transition from metarhodopsin I to metarhodopsin II is accompanied by the uptake of a proton (Radding and Wald, 1956a; Falk and Fatt, 1966), and it is the first step in the photolytic process that requires water (Wald et al, 1950). This step is also characterized by a large positive change in entropy and a large entalpy of activation, larger than in the other steps of the photolytic process (Abrahamson and Ostroy, 1967). This transition is thought to trigger visual excitation (see 1.3).

Above 0°C metarhodopsin II in detergent solution is slowly hydrolyzed to retinaldehyde and opsin. Some investigators believe that another intermediate occurs in this reaction. Matthews et al (1963) reported a product that was formed spontaneously in the dark at 3°C from metarhodopsin II. This compound absorbed maximally at 465 nm, was called pararhodopsin and considered to be a side product. However, Ostroy et al (1966 a) assumed pararhodopsin (also indicated as metarhodopsin III) to be a real intermediate, which is formed from metarhodopsin II and is then converted via N-retinylidene opsin into retinaldehyde and opsin.

Meanwhile photolysis of rhodopsin has also been investigated in situ. The photolytic process has been studied in excised eyes (Hagins, 1956), isolated retinas (Matthews et al, 1963; Baumann, 1968; Donner and Reuter, 1969) or rod outer segment suspensions Bridges, 1962; Pratt et al, 1964). These studies showed no differences with the results obtained for isolated rhodopsin preparations, except that the final product of the photolysis in situ appears to be retinol (Matthews et al, 1963) instead of retinaldehyde, which is the terminal product upon illumination of rhodopsin in detergent solution.

1.2.3. *Structure of rhodopsin*

A relationship between Vitamin A and visual pigments was indicated by the following observations. Fridericia and Holm (1925) reported that in the retina of vitamin A-deficient rats rhodopsin was formed more slowly than in normal rats. Addition of retinal tissue to the diet of rats suffering from vitamin A deficiency had a curative effect (Holm, 1929). Wald (1933; 1935) showed that vitamin A could be extraced from illuminated frog retinas. Extraction of dark adapted retinas with chloroform bleached the visual pigment and yielded not vitamin A, but a new compound which he called "retinene".

Ball et al (1948) identified retinene as vitamin A aldehyde or retinaldehyde Hubbard and Wald (1951) succeeded shortly afterwards in the preparation of rhodopsin from retinaldehyde and opsin (the name opsin was given to an illuminated rhodopsin preparation from which the retinaldehyde was removed by hexane extraction) These and other experiments strongly support the idea that retinaldehyde is the chromophoric group of rhodopsin. Oroshnik et al (1956) presented evidence that out of the many possible retinaldehyde isomers only the 11-cis isomer could react with opsin with the formation of rhodopsin, suggesting that the 11-cis isomer is present in rhodopsin The retinaldehyde isolated from illuminated rhodopsin appeared to be the all-trans isomer, (Hubbard and Wald, 1952), indicating that illumination of rhodopsin causes isomerisation of the retinaldehyde from the 11-cis to the all-trans configuration In agreement with this conclusion was the finding that 11-cis retinaldehyde could be isolated from thermally denaturated rhodopsin (Hubbard and Kropf, 1958, Hubbard, 1958a, Hubbard, 1958b)

Rhodopsin is part of the lipoprotein complex forming the membranes of the rod sacs (1 2) The protein character of the visual pigments was already noted in 1878 by Ewald and Kuhne and was afterwards confirmed by a large number of investigators (Bridges, 1970a) Amino acid analyses performed on purified rhodopsin preparations from various animal species have been reported by Shields et al (1967) (cattle), Heller (1968a, 1969), (cattle, frog and rat) and Shichi et al (1969), cattle). The reported amino acid compositions differed to a certain extent, suggesting that not completely pure rhodopsin preparations were used In spite of this, agreement was reached for the molecular weight of the protein part of rhodopsin Shields et al, (1967) 28,600, Heller (1968a, 1969) 27,000 - 28,000, Shichi et al (1969) 28,000 Krinsky (1958) reported a value of 18,000 for the protein part of cattle rhodopsin calculated from the protein nitrogen content of the rhodopsin preparation These values are much lower than the value of 40,000 previously obtained by Hubbard (1954) who measured the apparent molecular weight of rhodopsin-digitonin micelles in the analytical ultrcentrifuge This high value is probably due to the presence of substantial amounts of lipids in these extracts (Krinsky, 1958, Adams, 1969)

On dry weight basis 30-40^{o}/o of cattle or frog rod sac membranes consist of phospholipids (compare Chapter 3, Table I). An interesting question is whether all these phospholipids are essential for the integrity of the rhodopsin

complex. Lythgoe (1937) reported that extraction of rhodopsin by homopolar solvents like petroleum ether or hexane does not affect the absorption characteristics and photolytic properties of the visual pigment. This extraction removes half of the phospholipids originally present in the outer segment membranes (Borggreven et al, 1970). Treatment of rod outer segments with phospholipase C from Clostridium perfringens enabled Krinsky (1958) to remove 80°/o of the phospholipids originally present without damaging the rhodopsin. So by far the largest part of the phospholipids in the rod sac membranes does not seem to be essential for the spectral integrity of rhodopsin.

1.2.4. *Structure of the rod sac membrane*

Several investigators have studied the problem of how the rhodopsin molecules are located in the saccular membranes. Electronmicroscopic investigation of these membranes showed the triple layered structure as found in other cellular membranes (Sjöstrand, 1961; De Robertis and Lasansky, 1958). Fernandez-Moran (1962) reported however the presence of globular structures in frog rod sac membranes. This observation was confirmed some years later by the refined electronmicroscopic observations of Nilsson (1964, 1965). Nilsson observed the presence of globular subunits with a diameter of about 25 Å in cross-sections of the disc membranes. Negatively stained (phosphotungstate or permanganate) frog disc membranes showed in surface-on view particles with a diameter of about 40 Å.

X-ray studies were undertaken, which did not require chemical treatment of the membranes. Low angle X-ray diffraction patterns from frog saccular membranes indicated the presence of globular particles, distributed in a non-crystalline liquid-like way in the plane of the membrane (Blasie et al, 1969; Blasie and Worthington, 1969). The diameter of these particles was estimated to be about 44 Å. The effect of antirhodopsin serum (Dewey et al, 1969) on the X-ray diffraction patterns, indicates that these particles are protein particles containing the visual pigment rhodopsin (Blasie et al, 1969). The diameter of a spherical protein with a molecular weight of 28,000, which equals the molecular weight of rhodopsin in various vertebrates, would indeed be about 40 Å, assuming a protein density of 1.33 g/cm^3. From gelfiltration

of detergent solutions of frog, cattle and rat rhodopsin, Heller (1969) derived a Stokes diameter of 46 Å.

On the basis of these results Vanderkooi and Sundaralingam (1970) have proposed a model for the saccular membrane (Fig.4). In this model the protein particles, representing rhodopsin, and the phospholipid molecules together form a bilayer, rather than just the lipids as is proposed in the classical

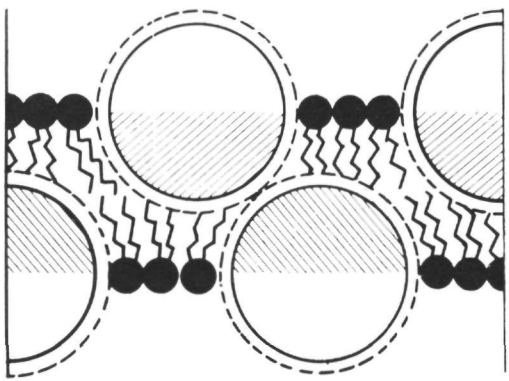


Figure 4. Molecular arrangement of the rod sac membrane as proposed by Vanderkooi and Sundaralingam (1970).
The black circles are the polar heads of the phospholipid molecules and the crooked lines represent their non-polar fatty acids. The large circles are the proteins, with the solid line representing a 40 Å sphere and the dashed line a 46 Å sphere. The cross hatched parts of the proteins are relatively non polar and interact hydrophobically with the phospholipids.

Danielli-Davson membrane model. The lipids themselves are also in the bilayer arrangement, filling up the space between the protein molecules.

Experiments with polarized light indicate that the visual pigment molecules are oriented in the rod sac membranes in such a way that the chromophoric retinaldehyde group lies in the plane of the rod sac surface (Schmidt, 1938; Liebman, 1962; Wald et al, 1963). This position is the most favourable for the absorption of the incident light beam, which is perpendicular to the disk surface. In the Vanderkooi-Sundaralingam model the protein units are

thought to "float" with the least polar part in the lipid field of the membrane, while the other half of the molecule is in the waterphase surrounding the membrane. This would orient the proteins in such a way that the associated retinaldehyde molecules would always be orientated in the same way relative to the incident light.

1.3. VISUAL EXCITATION

A problem in vision which has not been solved until now is how molecules of the visual pigment, having absorbed incident radiation, can initiate a nervous response in the eye. This is the problem of the transduction mechanism. Hecht et al (1942) showed that a rod may be stimulated by absorption of a single light quantum. This means that a considerable amplification is involved between absorption and excitation. This poses the problem of the amplification process. For this transduction-amplification mechanism three hypotheses have been proposed: the enzyme hypothesis, the solid state hypothesis and the ionic hypothesis.

The enzyme hypothesis (Wald, 1956) postulates that rhodopsin is a proenzyme which is converted into an enzymatically active conformation by illumination. The conversion of one enzyme molecule would cause the transition of a large number of substrate molecules, thus causing a biochemical amplifying system.

The solid state hypothesis (Wald et al, 1963) is based on the highly ordered structure of the outer segment, also on the molecular level. Excitation of one molecule of rhodopsin would then stimulate many other molecules in its neighbourhood by resonance. Or alternatively, photoconduction would take place, due to electrons which are brought into a conduction band by the action of light.

The ionic hypothesis (Bonting and Bangham, 1967) is based on experimental evidence for the existence of a cationic gradient across the rhodopsin containing saccular membrane. Absorption of light by a rhodopsin molecule would cause an increase in cationic permeability of the saccular membrane, thus causing a sudden passive cationic flux along the gradients existing across this membrane. The resulting cation currents would be responsible for the excitation of the synaptic end of the rod cell.

The enzyme and solid state hypotheses are supported by too little experimental evidence to give them real significance By far the most support has been reported for the ionic hypothesis from electrophysiological investigations as well as from studies on the cationic composition of rod outer segments Arguments pro and con each of the three hypotheses have recently been reviewed (Bonting, 1969)

A problem in the ionic hypothesis is how photolysis of a rhodopsin molecule in the saccular membrane can affect the state of the outer membrane, such that ion fluxes take place This problem has been investigated by Bonting and Bangham (1967) These investigators observed that illumination of a rod outer segment suspension caused efflux of K^+ ions and an equivalent influx of Na^+ ions The same effect could be achieved by addition of all-trans retinaldehyde in the dark to the suspension All-trans retinaldehyde added to micelles of phosphatidyl ethanolamine liposomes greatly increased cation leakage through these micelles, but had no effect on lecithin micelles which do not possess free amino groups All-trans retinol or retinoic acid had no effect on either type of micelles (Bonting and Bangham, 1967, Daemen and Bonting, 1969) These results suggest that the aldehyde group of retinaldehyde is essential for the cation leakage effect and that amino groups play a role in this process Additional evidence for aldimine formation between retinaldehyde and an amino group of rhodopsin upon illumination of rhodopsin was obtained from studies with monolayers of rhodopsin and phospholipids in a Langmuir trough (Bonting and Bangham, 1967, de Pont et al, 1968)

On the basis of their findings Bonting and Bangham (1967) formulated the mechanism of visual excitation as follows Absorption of light leads to photolysis of rhodopsin The retinaldehyde in rhodopsin is thus liberated from its original bond to opsin and undergoes transiminization to another amino group in the rhodopsin-membrane complex The blocking of the latter amino group would induce a local negative charge on the saccular membrane The local negative membrane charge can lead to a sudden increase in cation permeability This would permit Na^+ ions to rush into and K^+ ions to leak out of the rod sac This would in their view cause a photoreceptor current which could activate the cholinergic mechanism at the photoreceptor bipolar synapse Subsequent recovery of the cationic gradients would be achieved by the sodium-potassium activated ATPase system located in the saccular mem-

brane This hypothesis appears to be in need of revision in view of the work of Penn and Hagins (1969) and of Sillman et al (1969) Penn and Hagins show evidence for a dark current by Na^+ ions moving across the outer membrane into the outer segment, which decreases upon illumination Sillman et al conclude that illumination causes a sodium permeability decrease in the outer membrane as well as a hyperpolarization across this membrane The as yet unsolved problem is how the photolytic event in the rod sac membrane can explain these findings for the events in the outer membrane

In concluding this section something may be said about the photolytic step responsible for visual excitation From the moment of light absorption to the moment of generation of the receptor potential takes about one millisecond This excludes any step beyond the formation of metarhodopsin II, since the decomposition of metarhodopsin II at room temperature takes several minutes The transition of rhodopsin to metarhodopsin I takes place within 4 μsecs at 0°, while the formation of metarhodopsin II takes about one millisecond at physiological temperatures (Ostroy et al, 1966 a) In view of the drastic changes in the metarhodopsin I $\longrightarrow$ II conversion (large spectral shift, requirement for water, proton uptake, large enthalpy and change in entropy), Bonting (1969) has suggested that this step is responsible for excitation In the Bonting-Bangham hypothesis this would then also be the photolytic step in which transiminization of retinaldehyde from its original binding site on opsin to an amino group in the saccular membrane The proposed transiminization of retinaldehyde from its original binding site to another site on the opsin molecule, has been the basis for our study on the presence and role of phospholipids in rhodopsin

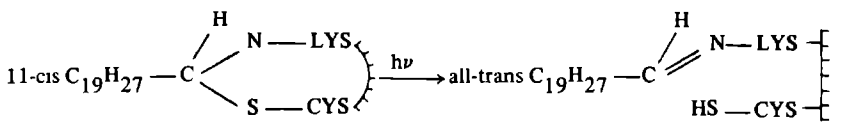
14. NATURE OF THE RETINALDEHYDE LINK

When one wants to know whether visual excitation would be caused by a transition of retinaldehyde from its original binding site to another, it is necessary to know how retinaldehyde is bound in rhodopsin, and secondly how it is bound in metarhodopsin II.

The work of Morton and his associates led to the idea that the aldehyde group of retinaldehyde in rhodopsin is linked to opsin by an aldimine linkage The most important experimental evidence for this suggestion came from

Collins (1953) and Morton and Pitt (1949). The latter investigators showed that the acid denaturation product of rhodopsin was N-retinylidene-opsin (C_{19} H_{27} CH = N^+H - opsin). Having found from model reactions that aldimine formation does not readily occur at acid pH, they concluded that the aldimine linkage had been present in rhodopsin itself before denaturation. Other evidence leading up to this conclusion has been summarized by Morton and Pitt (1957) and Dartnall (1962).

It has also been proposed that sulfhydryl groups are involved in the retinaldehyde-opsin linkage in rhodopsin, either in the place of a carbon to nitrogen bond or in addition to it. This idea comes from the observation that several extra titratable sulfhydryl groups occur upon illumination of rhodopsin and that regeneration of rhodopsin from 11-cis retinaldehyde and opsin is inhibited by sulfhydryl reagents like p-chloromercuribenzoate (Wald and Brown, 1952). A substituted aldimine linkage of retinaldehyde with lysine and cysteine has been proposed by Mizuno et al (1966a; 1966b) and by Heller (1968, b). The first photolytic step would then be as follows:



I II

The compact conformation of opsin in I would be converted by illumination to a more expanded conformation II. Heller (1968b) commented that this scheme would explain:

1. the liberation of one additional titratable sulfhydryl group upon illumination of rhodopsin,
2. the expansion of the opsin moiety (Heller, 1968 b) upon illumination,
3. the fact that the retinyl-opsin linkage becomes accessible to reduction with sodium borohydride after illumination of rhodopsin (Bownds and Wald, 1965),
4. the impossibility of regeneration of rhodopsin after the action of sulfhydryl reagents upon opsin.

An alternative explanation has, however, been suggested by Hubbard and Kropf (1959a), namely that through conformational changes in the opsin molecule during illumination the aldimine link of retinaldehyde to opsin as well as certain sulfhydryl group(s) on the protein would be uncovered. This would not require the assumption that a sulfhydryl group is involved in the retinaldehyde-opsin link.

The property of the aldimine C = N link that it can be reduced by $NaBH_4$ under neutral or weakly acid conditions to a secondary amine C - N link, which is much more stable, was used by Bownds and Wald (1956) to learn more about the retinaldehyde-opsin link. They noticed that native rhodopsin would only react with aqueous $NaBH_4$ when it was simultaneously illuminated. Peptic hydrolysis of the resulting reduced compound, presumably N-retinyl opsin, yielded a small peptide with the retinyl group attached to the ϵ-amino group of lysine (Bownds, 1967). This was independently confirmed by Akhtar et al (1967). Since simultaneous illumination of rhodopsin was necessary to achieve reduction with aqueous sodium borohydride, this proved only that in metarhodopsin II retinaldehyde is attached to the ϵ-amino group of lysine. Thus, the binding site of retinaldehyde in native rhodopsin was still open to speculation.

From his observations on rhodopsin incubated with phospholipase C, Krinsky (1958) suggested the possibility of an aldimine linkage between retinaldehyde and an amino group containing phospholipid. Adams (1967) reported the presence in a rod outer segment lipid extract of a new compound, possibly consisting of retinaldehyde linked to phosphatidyl ethanolamine or phosphatidyl serine. These observations and the hypothesis of Bonting and Bangham that in the metarhodopsin I $\longrightarrow$ II transition transiminization of retinaldehyde involving a phospholipid amino group would occur led Poincelot et al (1970) to a more detailed study. They extracted native unilluminated rhodopsin preparations with anhydrous methanol containing $2 \cdot 10^{-4}$M HCl. The acid served to protonate the aldimine link and thus avoid transiminization. They were able under those conditions to achieve virtually quantitative extraction of the chromophore as N-retinylidene phosphatidyl ethanolamine. They concluded from this that in native rhodopsin retinaldehyde should be bound to phosphatidyl ethanolamine. From a similar experiment with metarhodopsin I, obtained by bleaching dry rhodopsin, they concluded that the N-retinylidene phosphatidyl ethanolamine linkage is also present in meta-

rhodopsin I (Kimbel et al, 1970). For metarhodopsin II they confirmed the linkage to the ϵ-amino group of lysine, reported by Bownds (1967) and Akthar et al (1967). Transition of metarhodopsin I to metarhodopsin II would thus involve transiminization of the chromophore from phosphatidyl ethanolamine to lysine.

The presence of retinylidene phosphatidyl ethanolamine in rhodopsin was, however, disputed by other investigators. Heller (1968, a) purified rhodopsin in CTAB solution by means of gel filtration and found such a low phosphorus content in the lipid extract of this rhodopsin preparation that the presence of a linkage of retinaldehyde to a phospholipid in native rhodopsin seemed improbable. However, the accuracy of the lipid phosphorus determinations in the presence of a high detergent concentration and the purity of this rhodopsin preparation has been doubted by other investigators (Poincelot and Abrahamson, 1970a; Shichi, 1970). More recently, Hall and Bacharach (1970) measured the incorporation of ^{32}P-orthophosphate in rod outer segment lipids in vivo and concluded that the radioactivity present in a purified rhodopsin preparation was insufficient to account for a phosphatidyl ethanolamine-retinaldehyde link in native rhodopsin. Here, the implicit assumption of an equal ^{32}P turnover in free and in a retinaldehyde- bearing phosphatidyl ethanolamine left some doubt as to the validity of their conclusion. Very recently Anderson (1970) and Anderson and Maude (1970) reported that the molar amount of N-retinylidene phosphatidyl ethanolamine in an acidified chloroform-methanol extract of outer segment preparations was less than that of rhodopsin originally present in these preparations, suggesting that the phospholipid aldimine did not serve as the chromophore of rod visual pigment. However, only two rod preparations were analyzed giving molar ratios between N-retinylidene phosphatidyl ethanolamine and rhodopsin of 0.90 and 0.46 respectively for a molar absorbance of rhodopsin of 42,000.

1.5. PURPOSE AND SCOPE OF OUR INVESTIGATIONS

The uncertainty about the role of the phospholipids in the binding of retinaldehyde in native rhodopsin and thus in the mechanism of visual excitation (Bonting, 1969) led us first of all to a quantitative study of the phospholipids present in cattle rod outer segments. From the results of these analyses

the molar ratio of the amino group containing phospholipids (phosphatidyl serine and phosphatidyl ethanolamine) to the rhodopsin-bound retinaldehyde could be calculated. Since these were much larger than the minimal value of one, required for a phospholipid binding site of retinaldehyde, we decided to study the possibility of removal of phospholipids from the outer segment without destroying the spectral integrity of the visual pigment.

For this purpose hexane extraction of the rod outer segments was applied first. This removed only about half of the phospholipids, but the molar ratio between phosphatidyl ethanolamine or phosphatidyl serine and retinaldehyde was still considerably higher than one.

Since organic solvents, which extract lipids more effectively, denature rhodopsin, the action of phospholipases was tried. Incubation of rhodopsin with phospholipase C reduced the molar ratio between phosphatidyl ethanolamine and retinaldehyde considerably below 1. This allowed us to exclude phosphatidyl ethanolamine as the binding site for retinaldehyde in rhodopsin. However, not enough phosphatidyl serine was removed by phospholipase C to permit the same conclusion for this phospholipid.

It was then found that incubation of the phospholipase C treated preparation with phospholipase A and subsequent extraction of the resulting lysophosphatidyl serine with bovine serum albumin removed most of the remaining phosphatidyl serine, so that the existence of an aldimine linkage between this phospholipid and retinaldehyde in rhodopsin could also be excluded. From these results the complete absence of an aldimine linkage between retinaldehyde and a phospholipid in rhodopsin was concluded.

In view of an existing controversy concerning the true value of the molar absorbance coefficient of rhodopsin, which is a fundamentally important parameter for the determination of the rhodopsin concentration, we also made a critical study of its estimation. Our results confirmed the traditional high value of Wald and others, and not the low value claimed by Heller (1968a). Finally, as an additional parameter of the phospholipid denuded rhodopsin preparations their regenerating capabilities have been determined.

CHAPTER 2

THE MOLAR ABSORBANCE COEFFICIENT OF RHODOPSIN

2.1. INTRODUCTION

In our study of the relationship between phospholipids and rhodopsin in the cattle rod outer segment, an accurate and simple assay for rhodopsin was important. Rhodopsin can be determined spectrally from the height of the α-peak of rhodopsin at 500 nm (1.2.1), provided the value of the molar absorbance coefficient of rhodopsin at this wavelength is known. There exists, however, at present disagreement concerning the latter value in the literature.

The first value for the molar absorbance coefficient of cattle rhodopsin was reported by Wald and Brown (1953). They illuminated a rhodopsin sample of known 500 nm absorbance in the presence of hydroxylamine to convert the all-trans retinaldehyde being liberated quantitatively into the form of its oxime. Having determined the molar absorbance coefficient of pure all-trans retinylidene oxime at 360 nm, they calculated for rhodopsin a molar absorbance coefficient of 40,600 L/mol.cm from the ratio of the absorbance at 500 nm before and at 360 nm after photolysis. They assumed that one retinaldehyde molecule was present per molecule of rhodopsin, an assumption which has subsequently been shown to be correct (Hubbard, 1954; Heller, 1968b). Futterman and Saslaw (1961) confirmed the value of Wald and Brown (1953) by determining retinaldehyde in retina and in rod outer segment preparations by means of a thiobarbituric acid assay. Recently Heller (1968a) reported a much lower value of 23,100, which he calculated from the absorbance of a rhodopsin sample at 500 nm, its amino acid composition and a molecular weight for the protein part of 26,400 assuming that the preparation was pure rhodopsin. Subsequently Shichi et al (1969) arrived at a value of 42,000 from the absorbance of purified rhodopsin and a retinaldehyde determination in the same sample by the thiobarbituric acid method. Later he (Shichi, 1970) confirmed this value by means of the method of Wald and Brown (1953). Heller (1970) commented that the thiobarbituric acid method gave "only very erratic and non-reproducible results with visual pigments, though excellent reproducibility and accuracy were obtained with free all-trans retinaldehyde".

We have re-examined the problem by application of a slightly modified form of the method of Futterman and Saslaw (1961) on isolated rod outer segments. The results of this study are described in this chapter, and have also recently been published (Borggreven et al, 1970; Daemen et al, 1970).

2.2. METHODS AND MATERIALS

2.2.1. *Isolation of cattle rod outer segments*

Cattle eyes were stored in a light-tight container for 1-2 hr after death at room temperature before further treatment. The 1-2 hr period at room temperature in darkness served to achieve maximal regeneration of any bleached rhodopsin present in the eyes. The retinas were then dissected out in the following manner. An incision was made at the ora serrata to remove the cornea. The lens and vitreous body were then expelled easily by light pressure on the eye ball. Two incisions of about 3/4 cm were made at the edge of the eyecup, which was then turned inside out. The retina was loosened cautiously and finally the connection with the nervus opticus was cut.

Retinae from 60-80 dark adapted cattle eyes were briefly homogenized in saline (0.5 - 1 ml per retina) in a Potter-Elvehjem homogenizer by moving the loosely-fitting teflon pestle (clearance 1.8 mm) slowly up and down 40 times to detach the outer segments from the rest of the retina (McConnell, 1965). The homogenate was then filtered through 120-mesh stainless steel wire screen. The residue on the gauze was resuspended in 25 ml saline, homogenized and filtered again. The filtrates were combined and the washing procedure was repeated. The combined filtrate was then mixed with 66.7°/o (w/w; 2.52 M) aqueous sucrose to a final concentration of 0.42 M. From this suspension and an aqueous 1.28 M sucrose solution continuous gradients with a density range of 1.05 - 1.16 (0.42 - 1.28 M) were prepared in four 45 ml centrifuge tubes. Centrifugation at 20,000 x g (Sorvall HB-4 rotor) yielded, in the middle of the tubes, a purple layer containing rod outer segments. (Occasionally, instead of one layer two adjacent layers with identical spectral properties were obtained). The rod outer segment layer was then isolated by suction with a pipette and diluted with 5 volumes saline. The insoluble material was collected by centrifugation (15 min at 18,100 x g Sorvall SS-34

rotor), washed and resedimented twice in distilled water. The final sediment was generally lyophilized. One retina yielded 1 - 1.5 mg of lyophilized rod outer segment membranes. All manipulations were carried out in dim red light or darkness.

2.2.2. *Reference compounds*

All-trans retinaldehyde was purchased from Eastman Kodak Co., Rochester, New York.

A pure preparation of 11-cis retinaldehyde was obtained in our laboratory by drs. J.P. Rotmans from 11-cis retinoylacetate (kindly supplied by Hoffmann-La Roche, Basel, Switzerland) in the following manner. One gram of 11-cis retinoylacetate was hydrolyzed by shaking it for 1.5 hours with 10 ml of 10°/o methanolic potassium hydroxide at 20°C. The mixture was then diluted with 10 ml H_2O and vigorously shaken for one minute with 10 ml diethyl ether. After allowing the two layers to separate, the ether layer was collected and the water layer shaken again with fresh ether. The ether-extraction was then repeated once more. To the combined ether extract 10 g manganese dioxide was added and the mixture was shaken for 12 hours in order to oxidise the 11-cis retinol to 11-cis retinaldehyde (Ball et al, 1948). The manganese dioxide was then removed from the suspension by centrifugation at 800 x g for 5 minutes. The supernatant solution was evaporated and the 11-cis retinaldehyde taken up in hexane. This solution was stored at -70°C. All manipulations were carried out under nitrogen in the dark or in dim red light at temperatures not exceeding 20°C.

The all-trans retinaldehyde as well as its 11-cis isomer appeared to be pure (> 95°/o) when investigated by chromatography on silicagel (development with hexane-diethylether 85 : 15, by vol.; detection of spots by spraying with a 5°/o $SbCl_3$ solution in chloroform).

2.2.3. *Spectral determination of rhodopsin*

Dry rod outer segment preparations were homogenized in 67 mM phosphate buffer pH 6.8 containing 1°/o Triton X-100 or 1°/o digitonin (1 mg

rod preparation/ml). After extraction for 10 min (Triton X-100) or one hour (digitonin) the suspension was centrifuged for 5 min at 18,100 x g. Then 10 µl of 1M aqueous hydroxylamine, adjusted to pH 6.8, was added to 200 µl of the supernatant solution. The absorption spectra of the rhodopsin extracts were measured before and after exhaustive photolysis by a 10-min illumination with a 75 W tungsten lamp at a distance of 20 cm through ultraviolet and infrared filters (KG-1 and GG-3 filters, thickness 3 mm each, Schott-Jena, Mainz, Germany). The difference in absorbance of these extracts at 500 nm before and after photolysis is designated as ΔA_{500}/mg and is a quantitative measure of the amount of rhodopsin present.

2.2.4. *Determination of retinaldehyde*

Retinaldehyde was determined by means of a modification of the thiobarbituric acid method of Futterman and Saslaw (1961). The thiobarbituric acid reagent was prepared by dissolving 600 mg of thiobarbituric acid in 100 ml absolute ethanol. The solution was filtered and stored in a refrigerator. The thiourea reagent was prepared by dissolving 4 g thiourea in 100 ml of glacial acetic acid. The solution was filtered and stored at room temperature.

Samples of lyophilized rod outer segments (1 - 2mg) were placed in Potter-Elvehjem tubes which could be centrifuged. After addition of 100 µl propanol and 1 ml of a freshly prepared mixture of thiobarbituric acid and thiourea reagents (1:1, by vol.) the mixture was homogenized, allowed to stand for 30 min, homogenized a second time and centrifuged for 10 min at 18,100 x g. The absorbance of the supernatant was measured at 530 nm. The assay was standardized against a 0.1 mM solution of all-trans retinaldehyde in propanol.

In cases where the recovery of retinaldehyde added to the rod preparations was determined, the initially added 100 µl propanol was replaced by 100 µl of an all-trans retinaldehyde solution of known concentration in propanol. The retinaldehyde determination was then carried out in the normal manner.

Unless indicated otherwise all manipulations were carried out in dim red light or in darkness.

2.2.5. *Preparation of metarhodopsin I*

Dry rod outer segment preparations were spread in a thin layer over the walls of Potter-Elvehjem tubes. The preparations were then illuminated in the dry state for 30 min by a 75 W tungsten lamp at a distance of 15 cm through ultraviolet and infrared filters (KG-1 and GG-3 filters, thickness 3 mm each, Schott-Jena, Mainz, Germany). Metarhodopsin I formation was indicated by a colour change from purple to orange.

2.3. RESULTS

2.3.1. *Isolation of cattle rod outer segments*

Rod outer segments were isolated from a retinal homogenate on a continuous sucrose gradient ranging from 0.42 - 1.28 M. After centrifugation of the gradient two bands were observed (Fig.5). The lower one (band II) was pale yellow, while the upper one (band I) had a reddish-purple colour and contained the rod outer segments as shown by the disappearance of the reddish colour upon illumination. The precipitate present on the bottom (fig.5, band III) had a black colour due to the presence of pigment granules. These granules originate from the pigment epithelium, which in the living eye is in close contact with the photoreceptor cells. The purity of the rod outer segments in band I of the gradient was checked by light and electron-microscopic observations. Contamination with other cell particles was found to be negligible (Fig.6). The purity of the material, especially with respect to mitochondrial contamination, was further confirmed by the very low content of diphosphatidyl glycerol (0.2°/o of the phospholipids present in the rod outer segment preparations, Chapter 3, Table III), which is in general a major component in mitochondria (Fleischer and Rouser, 1965).

2.3.2. *Determination of rhodopsin*

The amount of rhodopsin present in the rod outer segment preparations was characterized by the value of the ΔA_{500} per mg (2.2.3). This value

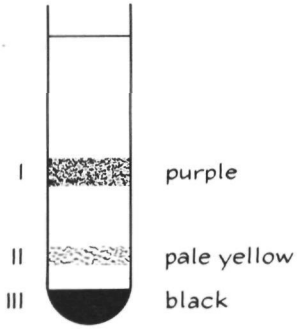


Figure 5. Schematic drawing of the sucrose gradient (0.42 - 1.28M) used for the isolation of rod outer segments after centrifugation. For further explanation, see text.

Figure 6. Cattle rod outer segments isolated as described in the text. Fixation by glutaraldehyde followed by osmium tetroxide, staining with uranylacetate and lead citrate. Magnification, 4600 x.

varied in Triton X-100 extracts of twenty-three different rod preparations from 0.150 to 0.250 (fig.7), with a mean of 0.206 (S.E. 0.008). The molar amount of rhodopsin was calculated from ΔA_{500} and the molar absorbance coefficient.

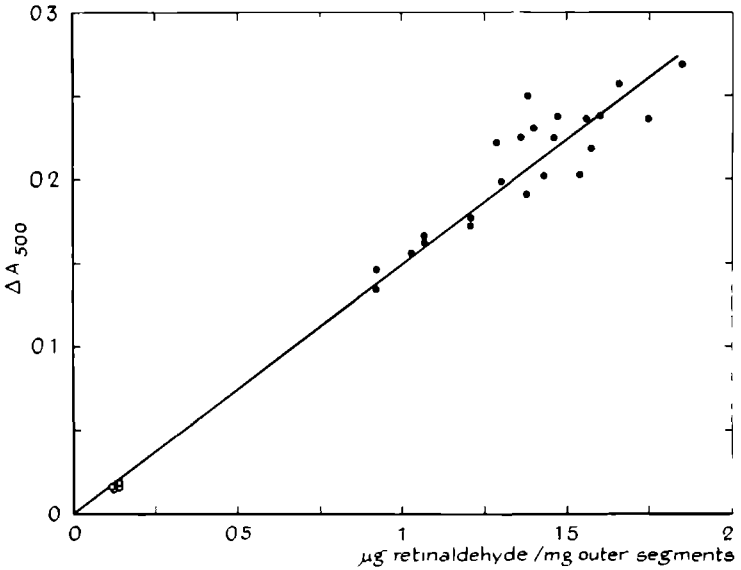


Figure 7. Correlation between ΔA_{500}/ mg and retinaldehyde content of twenty-three cattle rod outer segment preparations.
The four points in the lower left represent values obtained from whole homogenized, lyophilized cattle retinas.

2.3.3. *Determination of retinaldehyde*

Retinaldehyde present in the rod outer segment preparations was determined by means of a slightly modified method of Futterman and Saslaw (1961). As shown in Fig.8, a linear relationship exists in this assay between the amount of retinaldehyde and the absorbance at 530 nm. Futterman and Saslaw (1961) showed that retinaldehyde could be effectively extracted from

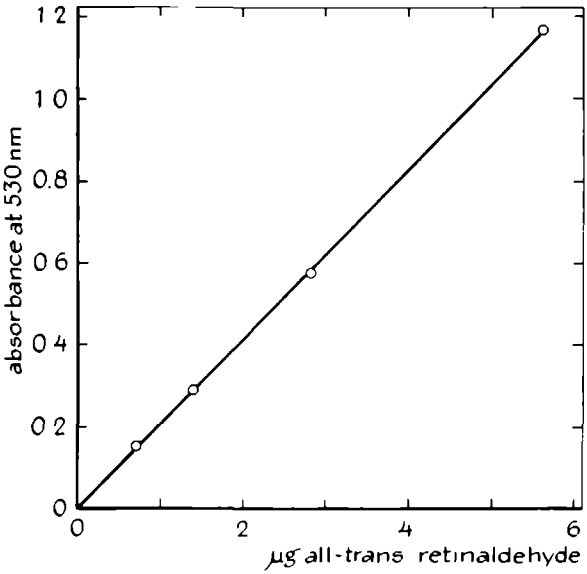


Figure 8. Relationship between the amount of all-trans retinaldehyde and the absorbance at 530 nm in the thiobarbituric acid assay.

rod outer segments with 90°/o ethanol. Aliquots of these extracts were then quantitatively determined with the thiobarbituric assay. We found that pretreatment with ethanol was not essential since the propanol-thiobarbituric acid-thiourea mixture extracts the retinaldehyde from the rod material to the same extent. In three different outer segment preparations our direct method without ethanol extraction gave 1.05 (S.E. 0.05) times the value obtained by the original Futterman-Saslaw method with ethanol extraction. The principal advantage of our method is that an amount of 0.25 μg retinaldehyde can be determined with reasonable accuracy, which is one sixth of that necessary in the Futterman-Saslaw method. In addition the number of manipulations is decreased through the omission of the extraction of retinaldehyde by ethanol. For twenty-three rod outer segment preparations we found a retinaldehyde content varying from 1.0 - 1.7 μg retinaldehyde per mg with an average value of 1.37 (S.E. 0.05) μg / mg. The reproducibility of the simplified method was 5°/o, when carried out in routine fashion.

All retinaldehyde determinations were standardized against all-trans retinaldehyde. In native rhodopsin, however, retinaldehyde is in the 11-cis configuration. In earlier reports it was not always clear whether or not the rhodopsin preparations had been subjected to photolysis before the retinaldehyde assay. This could cause an error in the molar absorbance value, if all-trans and 11-cis retinaldehyde would have a different chromogenicity towards the thiobarbiturate - thiourea reagent. This possibility is unlikely, since we found the same results, regardless of whether all procedures, including the retinaldehyde assay, were carried out in dim red light or in normal room light. However, we have explicitly tested this possibility by comparing the chromogenicity in the thiobarbituric acid assay before and after conversion of rhodopsin by illumination in the dry state to metarhodopsin I, which contains all-trans retinaldehyde as the chromophoric group. For four different outer segment preparations the chromogenicity of the samples in the metarhodopsin I state was 97.4°/o (S.E. 2.6) of that in the rhodopsin state. We further investigated the chromogenicity of the retinaldehyde isomers by comparing the pure 11-cis retinaldehyde with all-trans retinaldehyde. Fig.9 shows

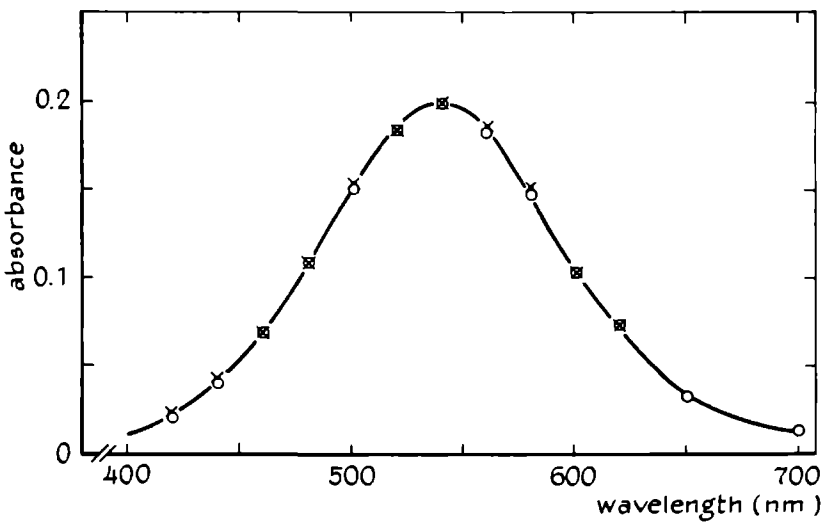


Figure 9. Absorption spectrum of the chromogens formed by all-trans (x —— x) and 11-cis (o—— o) retinaldehyde in the thiobarbituric acid assay.

that the chromogens formed by the two isomers had identical absorption spectra with an absorption peak at 530 nm. The colour intensity of the chromogen of 11-cis retinaldehyde was 95.1°/o (S.E. 3.9°/o, n = 2) compared to that for the all-trans retinaldehyde. We therefore concluded that all-trans and 11-cis retinaldehyde show equal chromogenicity in the thiobarbituric acid method, as was previously suggested by Futterman and Saslaw (1961) and very recently confirmed by Zorn and Futterman (1971).

The recovery of retinaldehyde added to rhodopsin was also checked since Heller (1970) suggested that the variability and low yield of the thiobarbituric acid reaction, which he found when applying it to rod outer segment preparations, might arise from a reaction of retinaldehyde with free amino groups of the protein component of rhodopsin. Known amounts of retinaldehyde in propanol were pipetted into Potter-Elvehjem tubes, a weighed sample of outer segment preparation was added and the assay was carried out as described in 2.2.4. For seven preparations thus investigated the recovery of the added retinaldehyde was 103°/o (S.E. 4°/o), indicating that there was no loss through interaction between the added retinaldehyde and the free amino groups in rhodopsin.

2.3.4. *The molar absorbance coefficient of rhodopsin*

The molar absorbance coefficient of rhodopsin at 500 nm is defined by the equation

$$A = \epsilon.c.d.$$

in which A is the absorbance of the solution at 500 nm due to the presence of rhodopsin, ϵ the molar absorbance coefficient, c the concentration in moles rhodopsin per litre and d the depth of the solution in centimeters. When the depth of the solution is known, it is possible to calculate ϵ from the values of c and A. A is determined from the ΔA_{500}/mg for a preparation. Since one mole of rhodopsin contains one mole of retinaldehyde (Hubbard, 1954; Heller, 1968 b), c can be calculated from the retinaldehyde content of a preparation on a molar basis.

The correlation between the ΔA_{500}/ mg and the retinaldehyde content of twenty-three preparations is illustrated in fig.7. The correlation coefficient is 0.90 ($P < 0.001$). The intercept of the regression line ($y = 0.022 + 0.135\ x$) is not significantly different from zero (Student intercept $t_{21} = 1.10$; $P > 0.25$). Thus, straight proportionality exists between the ΔA_{500} / mg and the retinaldehyde content of the outer segments. This is further illustrated by the four points in the lower left of the figure, which represent values for whole retina and fall close to the line.

From the average ΔA_{500} / mg and retinaldehyde content we calculated a molar absorbance for cattle rhodopsin of 43,000 l/mole. cm (S.E. 700, $n = 23$).

2.4. DISCUSSION

The value of 43,000 l/mol.cm obtained by us for the molar absorbance coefficient of rhodopsin at 500 nm agrees with the values of 40,600, 40,600 and 42,000 reported respectively by Wald and Brown (1953), Futterman and Saslaw (1961) and Shichi et al (1969). A common point in these investigations is the calculation of the molar absorbance coefficient from the molar retinaldehyde content (determined by direct assays or by determination of the amount of oxime formed upon photolysis of rhodopsin in the presence of hydroxylamine) and the absorbance at 500 nm of a rhodopsin preparation.

The deviating value of 23,100 reported by Heller (1968a) was obtained in a different way. He purified rhodopsin in a solution of cetyltrimethyl ammonium bromide by means of gel filtration and estimated the molecular weight of the purified rhodopsin in detergent solution by elution from a calibrated agarose column. A more precise value of the molecular weight was then calculated from the results of the quantitative amino acid analysis of the protein present in the rhodopsin preparations. The molar absorbance coefficient of rhodopsin was calculated from the absorbance at 500 nm and the molar amount of rhodopsin, which in turn was computed by dividing the amount of protein present on a weight basis by the molecular weight of the protein, assuming that all protein present in the preparations was part of the rhodopsin complex.

The discrepancy existing between the low value of Heller and the high

values reported by others and ourselves could be explained in two different ways: through a low retinaldehyde value in our assay, or through contamination of Heller's rhodopsin preparation by other proteins. Heller (1970) suggests the first possibility, namely that the retinaldehyde determination in rhodopsin preparations yields low results through aldimine formation between retinaldehyde and free amino groups of the protein present. In 2.3.3. we reported that this type of interference does not occur in our determination. Neither is it a disturbing factor in the type of retinaldehyde determination applied by Wald and Brown (Bridges, 1970b). The second possibility would be that Heller's purified rhodopsin preparations were contaminated by other protein(s). Such impurities would give a falsely high value for the molar amount of rhodopsin-protein present, leading to a falsely low molar absorbance coefficient. An index for the purity of a rhodopsin preparation is the A_{278} : A_{500} ratio. Upon purification of rhodopsin this value decreases. Heller reported a value of 1.6 for the A_{278}: A_{500} ratio, which lies close to the value of 1.75 reported by Shichi et al (1969). However, Heller (1968a) corrected the absorption spectrum of rhodopsin for the non-specific absorption of the cetyl trimethyl ammonium bromide used to solubilize his rhodopsin preparation by linear extrapolation of the absorbance between 310 and 340 nm to the 280 nm range. Since this manipulation reduces the 278 nm extinction value (without correction A_{278} : A_{500} would be about 2.0), it is possible that Heller overestimated the purity of his preparations. It should be kept in mind that even rhodopsin with the lowest A_{278} : A_{500} value of 1.6 reported thus far may still contain an appreciable amount of non-rhodopsin protein. In addition Heller used a molecular weight value of 27,700 determined by gel filtration of rhodopsin in detergent solution against reference compounds. This method is of dubious validity because it presupposes that the detergent is bound to the various proteins proportionally to their molecular weights. The experimental evidence for this supposition is poor, and non-proportional binding of detergent molecules to various proteins has been reported (Davison, 1968). This factor may, however, not be too serious, since Shields et al (1967) and Shichi et al (1969) obtained values of 28,600 and 27,700 resp. by referring amino acid analysis results to retinaldehyde content. While it is difficult to give an entirely satisfactory explanation for Heller's low value, as long as no facts are known which cast doubt on the procedures used by Wald and Brown (1953), Futterman and Saslaw (1961), Shichi et al

(1969), Shichi (1970) and ourselves the higher value is intrinsically the more likely one.

2.5. SUMMARY

Retinaldehyde present in cattle rod outer segment preparations, isolated by means of a continuous sucrose gradient technique, was determined with a thiobarbituric acid assay. It was shown that the presence of proteins or other compounds in the preparation does not interfere with the retinaldehyde determination. The two isomers, 11-cis and all-trans retinaldehyde, give the same absorbance in the thiobarbituric acid assay.

The retinaldehyde content of the rod outer segment was found to be 1.37 μg/mg rod preparation. From the ΔA_{500}/mg and the retinaldehyde content of the rod preparations a value of 43,000 l/mol.cm was calculated for the molar absorbance coefficient of rhodopsin at 500 nm, confirming the earlier value reported by Wald and Brown (1953) and the more recent values reported by Futterman and Saslaw (1961) and Shichi (1970), while not confirming the low value of 23,100 reported by Heller (1968 a).

CHAPTER 3

THE LIPID COMPOSITION OF NATIVE AND HEXANE-EXTRACTED CATTLE ROD OUTER SEGMENTS

3.1. INTRODUCTION

The membranes of the vertebrate photoreceptor cell are, like all other biological membranes, essentially lipoprotein complexes (Krinsky, 1958). A considerable part of the membraneous material of the rod outer segment consists of the visual pigment rhodopsin (Wald, 1961). Whilst the protein nature of the visual pigment had already been noted by Ewald and Kühne (1878) it was only in 1941 that Broda reported the consistent presence of phospholipids in visual pigment extracts. Suggestions for an intimate association between phospholipids and rhodopsin in rod outer segments came from Ishimoto and Wald (1946), Adams (1967), Krinsky (1958), Poincelot and Zull (1969) and Poincelet et al (1970). Krinsky (1958) put forward the possibility of the existence of a Schiff base linkage between an amino group containing phospholipid and retinaldehyde in rhodopsin. Poincelot and Zull (1969) and Poincelot et al (1970) suggested a Schiff base linkage between retinaldehyde and phosphatidyl ethanolamine in rhodopsin. A role for such an aldimine linkage in the process of visual excitation was indicated by Bonting and Bangham (1967). These indications of the importance of phospholipids in the rhodopsin complex brought us to a detailed study of the phospholipids present in the cattle rod outer segment. We started this work with a qualitative and quantitave determination of the neutral lipids, phospholipids and fatty acids present in the outer segments.

In this chapter the results of this analytical study are described and compared with data of other investigators. In addition, the change in lipid composition by hexane extraction, which removes a considerable part of the lipids without changing the spectral properties of rhodopsin, is reported. The results have been published previously (Borggreven et al, 1970).

3.2. METHODS AND MATERIALS

3.2.1. *Isolation of rod outer segments*

The isolation of cattle rod outer segments was performed as described in 2.2.1.

3.2.2. *Lipid extraction*

Complete lipid extraction from a weighed amount of rod outer segment preparations was carried out essentially according to the method of Folch et al (1957). Instead of the chloroform-methanol mixture (2:1, by vol.) used by these investigators for the extraction of lipids from wet tissues, we used a chloroform-methanol - H_2O mixture (60 : 30 : 3.6) thus correcting for the amount of water that had been removed from our preparation by the lyophilization.

To achieve optimal lipid extraction, the lyophilized material was dispersed in the extractant by homogenizing it in a Potter-Elvehjem homogenizer with 1 ml solvent per 10 - 15 mg lyophilized material. The suspension was shaken for one hour and then centrifuged for 10 min at 6,000 x g. The supernatant was collected and replaced by fresh solvent and the sediment extracted again as described above. Three extraction periods of one hour with fresh solvent were sufficient for complete lipid extraction. The total lipid extract was washed with 0.2 vol. 0.1 M KCl according to the method of Folch et al (1957) and the washed lipid extract was concentrated by evaporation and dissolved in a known volume of benzene-ethanol (4 : 1, by vol.). The lipid extracts were stored at -20°C under nitrogen.

Polyphosphoinositides were extracted from the residue by means of a chloroform-methanol-conc. HCl mixture (200 : 100 : 1, by vol.) according to the method of Eichberg and Hess (1967).

Hexane extraction of the rod outer segment preparations was performed for three periods of one hour in the same manner as described for the chloroform-methanol extraction. The hexane extracts were combined, evaporated to dryness and immediately dissolved in a known volume of chloroform-methanol-H_2O (60 :30 : 3.6, by vol.). This solution was washed with 0.1 M KCl,

concentrated by evaporation, and dissolved and stored in benzene-ethanol as described above for the chloroform-methanol-H_2O extraction.

All extractions were carried out under N_2 and in dim red light or in darkness unless indicated otherwise.

3.2.3. *Reference compounds*

Phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine and diphosphatidyl glycerol were obtained from Applied Science Laboratories, State College, Pennsylvania, U.S.A. When relatively large quantities were desired, phosphatidyl choline was prepared from egg-yolk according to the method of Pangborn (1951). Phosphatidyl inositol was a gift from Dr. F.A. Exterkate, Department of Biochemistry, University of Nijmegen. Lysophosphatidyl ethanolamine and sphingomyelin were kindly supplied by Dr. R.M. Broekhuyse, Department of Ophtalmology, University of Nijmegen.

Lysophosphatidyl choline was prepared by hydrolysis of egg-yolk phosphatidyl choline with phospholipase A from Crotalus adamanteus (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) according to the method described by Broekhuyse (1969). Lysophosphatidyl serine was prepared from synthetic phosphatidyl serine. Twenty mg phosphatidyl serine were dissolved in 6 ml freshly distilled diethylether. Then 0.3 ml 0.2 M Tris-maleic acid buffer pH 7.2 containing 0.001 M $CaCl_2$ and 5 mg phospholipase A from Crotalus adamanteus was added. The mixture was incubated for 1.5 hr at 37^o and evaporated to dryness under vacuum. The residue was taken up in a chloroform-methanol mixture (2 : 1, by vol.) and the solution containing the lysophosphatidyl serine and fatty acids from the parent phospholipid was used as reference mixture for thin layer chromatography.

Synthetic phosphatidylethanolamine and phosphatidyl serine were prepared in our laboratory by Dr. F.J.M. Daemen according to the method of Daemen (1967) and De Haas et al (1964), respectively.

Cholesterol was obtained from Merck, Darmstadt, Germany.

3.2.4. *Quantitative two-dimensional thin-layer chromatography*

For the analysis of rod outer segment phospholipids we used a slightly modified two-dimensional thin-layer chromatographic system with high resolving capacity developed by Broekhuyse (1968). Chromatographic plates (20 x 20 cm) were coated with a 0.30 mm thick layer of purified (Broekhuyse, 1968) silicagel H (Merck, Darmstadt, Germany) which, prior to application, was mixed with 4°/o alkaline magnesium silicate in a semi-micro Waring Blendor. The plates were dried for one hour at 130°C before use.

Application of aliquots of the lipid extract on the thin-layer plates was performed with a micro syringe pipette which permits application of exactly known volumes of the lipid extract with minimal risk of damaging the silica layer. One phospholipid analysis was performed with 15 - 20 μg lipid phosphorus. A chloroform-methanol- 7 N ammonia mixture (90 : 54 : 11, by vol.) was used as solvent system for the first dimension. After this development the plate was dried for one hour under vacuum over conc. H_2SO_4. Then development in the second dimension was carried out with a chloroform-methanol-acetic acid-water mixture (90 : 40 : 12 : 2, by vol.). After this development the plate was dried for 5 min in the air in a hood and coloured with iodine vapour in order to make the lipid spots visible.

For determination of plasmalogens, a chloroform-methanol-7 N NH_4OH mixture (75 : 34 : 6, by vol.) was used as solvent system for the first dimension. After development the lipid track was sprayed with a solution of 5 mM $HgCl_2$ in 0.1 M acetic acid which causes a splitting of the vinyl ether linkage present in plasmalogens, resulting in the formation of the corresponding lysophosphatides. After drying for one hour under vacuum over conc. H_2SO_4, the plate was developed in the second dimension with chloroform-methanol-acetic acid-water (75 : 25 : 8 : 3, by vol.).

3.2.5. *Staining procedures*

Iodine vapour was used for the detection of lipids (Owens, 1964). Phosphate esters were stained with the molybdate reagent of Dittmer and Lester (1962). Vicinal OH-groups were detected with the periodate-Schiff reagent (Baddiley et al, 1956). The Dragendorf reagent (Skidmore and Entenman,

1962) was used for the detection of choline groups and ninhydrin for free amino groups (Skidmore and Entenman, 1962).

3.2.6. *Quantitative determination of phospholipids after thin layer chromatography*

After staining of the chromatogram with iodine vapour each spot was quantitatively scraped into a 25 ml tube. After digestion with 0.4 ml of a mixture of conc. H_2SO_4 - 70°/o $HClO_4$ (9 : 1, by vol.) the amount of phosphorus present in each spot was determined by means of a modified Fiske-Subbarow method (Broekhuyse, 1968). Two non-coloured areas were also scraped from the chromatogram to serve as blank determinations. The blank values were practically independent of the amount of silicagel present. Thus, two blank determinations per chromatogram were generally sufficient in spite of the different sizes of the various lipid spots.

3.2.7. *Other analytical procedures*

Total lipid present in the rod outer segment preparations was determined by drying an aliquot of the washed lipid extract to a constant weight, which was determined on the Cahn electrobalance, Model G.

Lipid phosphorus was determined in an aliquot of the washed lipid extract, containing 1 - 10 μg phosphorus, by means of a modified Fiske-Subbarow method after H_2SO_4 - $HClO_4$ digestion (Broekhuyse, 1968). In calculating the phospholipid content the average phosphorus content of the phospholipids was assumed to be 4.0°/o.

Cholesterol was quantitatively determined after saponification of the lipid extract with alcoholic KOH which removes interfering substances (Abell et al, 1952). Aliquots of these extracts containing between 20 and 180 μg cholesterol were subjected to analysis by a modified Liebermann-Burchard procedure (Lynch et al, 1964).

Preparation of fatty acid methyl esters for gas-liquid chromatography was carried out with borontrifluoride by the method of Morrison and Smith (1964). Hydrogenation of the methylated fatty acids was performed on a

10°/o palladiumcarbon catalyst. Fatty acid separation was carried out over a column of 15 or 10°/o diethylene glycolsuccinate on Gas Chrom P (Applied Science) at 180°C.

3.3. RESULTS

3.3.1. *Lipid composition*

The lipid composition of rod outer segments is given in Table I. The value of 31.5°/o phospholipids includes approximately 0.5°/o polyphosphoinositides (not analysed in detail) extracted by a chloroform-methanol-HCl mixture after the chloroform-methanol-water extraction. For a correct determination of total cholesterol, saponification of the lipid extract with alcoholic KOH has been reported to be necessary in order to remove interfering

TABLE I

LIPID COMPOSITION OF THE CATTLE ROD OUTER SEGMENT

Values are expressed as weight percentages of dry rod outer segments The values are averages with standard errors for a number (n) of different rod outer segment preparations.

	Wt. °/o of dry rod outer segments	n
Total lipid*	39.0 ± 1.2	4
Phospholipid**	31.5 ± 0.9	7
Cholesterol***	3.1 ± 0.1	3
Retinaldehyde ϕ	0.137 ± 0.005	23

* By weight of total lipid extract

** As lipid-P present in total lipid extract

*** By the Liebermann-Burchard method on saponified total lipid extract

ϕ See Chapter 2

substances (Abell et al, 1952). We found that this was indeed the case with lipid extracts of the rod outer segments. When the extracts were not saponified, the value for cholesterol was nearly twice as high as in the saponified extract. Control experiments with pure cholesterol (Merck, Darmstadt, Germany) showed that the amounts of cholesterol determined before and after saponification were equal. Analysis of the saponified extracts gave a total cholesterol content including cholesterol esters) corresponding to 3.1 °/o of the rod outer segments an a dry weight basis (Table I).

The fatty acid composition of the rod outer segment lipids, which was determined by gas-liquid chromatography of the methyl-esters of the fatty acids is given in Table II. The identity of the methylated fatty acids were

TABLE II

FATTY ACID COMPOSITION OF THE CATTLE ROD OUTER SEGMENT

Number of C atoms and double bonds	Fatty acids (Wt. °/o of total fatty acid)*
16	0.6 ± 0.1
16 : 0	19.4 ± 0.3
16 : 1	0.8 ± 0.1
17 : 0	0.6 ± 0.2
18 : 0	23.1 ± 0.4
18 : 1	6.4 ± 0.3
18 : 2	1.4 ± 0.1
20 : 4	6.0 ± 0.2
22 : 4 **	1.5 ± 0.4
22 : 5 **	1.2 ± 0.7
22 : 6	34.3 ± 0.8
Unidentified	4.7 ± 1.3

* Averages with standard errors for three different rod outer segment preparations.

** Tentatively identified.

confirmed by comparison of their retention time with that of a reference compound as well as by hydrogenation over a palladium-carbon catalyst which converted the unsaturated fatty acids into the saturated analogues.

3.3.2. *Phospholipid analysis of native rod preparations*

The phospholipids of the rod outer segments were quantitatively analysed by means of a two-dimensional thin-layer chromatographic method, the advantages of which have been described previously (Broekhuyse, 1969). One of these advantages is the high resolving capacity of the system due to application of an alkaline solvent system for development in the first dimension combined with an acidic mixture for the second dimension.

Figure 10 shows a typical thin-layer chromatogram of rod outer segment phospholipids. The spots on the chromatogram were identified by means of specific staining reactions (see 3.2.5), by comparison with a sheep heart lipid extract of known phospholipid composition and by the use of authentic reference compounds (see 3.2.3).

In the chromatogram shown in Figure 10 the plasmalogens are located at the same place as the diacyl analogues. However, by this method lysophosphatidyl ethanolamine and phosphatidyl inositol are separated. For determination of plasmalogens their vinyl-ether linkages were hydrolysed with an acidic $HgCl_2$ solution after development in the first dimension (see 3.2.4.). The solvent systems used for development of the chromatograms were in this case somewhat different from those used in the former procedure. As a result, the phosphatidyl inositol spots now showed a weakly positive reaction with the ninhydrin reagent, probably due to contamination with phosphatidyl ethanolamine.

Quantitative phospholipid analyses were facilitated by the use of purified silicagel for chromatography. Thus phosphorus present in each spot of the chromatogram could be determined in the presence of silicagel. This eliminates the need for elution of the lipids from the silicagel, thus reducing loss of lipid phosphorus to a minimum.

The composition of the phospholipids present in cattle rod outer segments is given in Table III. The major phospholipids appeared to be phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl serine. The only

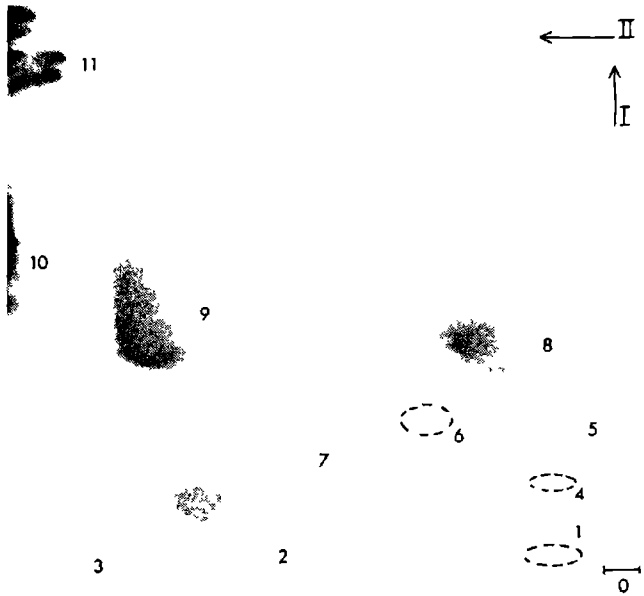


Figure 10. Two-dimensional thin-layer chromatogram of a total lipid extract of cattle rod outer segments.
Development in the first dimension with chloroform-methanol-7N ammonia (90·54:11, by vol.), in the second dimension chloroform-methanol-acetic acid-water (90.40.12:2, by vol.), staining with iodine vapour Identity of the spots: O, origin; 1, lysophosphatidyl serine; 2, phosphatidyl serine; 3, phosphatidic acid, 4, lysophosphatidyl choline; 5, sphingomyelin, 6, lysophosphatidyl ethanolamine; 7, phosphatidyl inositol; 8, phosphatidyl choline, 9, phosphatidyl ethanolamine; 10, disphosphatidyl glycerol, 11, retinaldehyde and other neutral lipids.

detectable plasmalogen was ethanolamine plasmalogen. To determine whether the phospholipid composition was unaffected by lyophilization of the rod outer segment preparation, phospholipid analysis was also carried out on fresh, non-lyophilized rod outer segments. No significant differences between the two preparations were observed

TABLE III

PHOSPHOLIPIDS PRESENT IN THE CHLOROFORM-METHANOL EXTRACT OF CATTLE ROD OUTER SEGMENTS BEFORE (A) AND AFTER HEXANE EXTRACTION (B) AND IN THE HEXANE EXTRACT (C).

The values are expressed as percentages of total lipid-P. The percentages with their standard errors are the means of duplicate analyses of several (n) rod outer segment preparations.

	A(n = 10)	B(n = 5)	C(n = 5)
Phosphatidyl ethanolamine	35.4 ± 0.7 (38.4)***	23.1 ± 1.2	51.0 ± 1.7
Ethanolamine plasmalogen	3.8 ± 0.2	1.7 ± 0.3	
Phosphatidyl choline *	34.7 ± 0.5 (32.9)	45.7 ± 1.5	21.0 ± 1.3
Phosphatidyl serine	11.2 ± 0.4 (12.2)	11.4 ± 0.8	13.0 ± 0.7
Phosphatidyl inositol **	5.7 ± 0.7 (6.0)	6.8 ± 1.0	5.3 ± 0.7
Lysophosphatidyl choline	1.7 ± 0.4 (1.9)	3.2 ± 1.0	0.7 ± 0.2
Sphingomyelin	1.0 ± 0.3 (0.8)	1.1 ± 0.4	0.6 ± 0.3
Diphosphatidyl glycerol	0.2 ± 0.1 (0.8)	0.6 ± 0.4	1.0 ± 0.4
Phosphatidic acid	0.4 ± 0.2	-	-
Others	2.3 ± 0.4 (1.7)	1.7 ± 0.6	1.8 ± 0.6
Recovery	96.4 ± 2.4	95.3 ± 2.2	94.4 ± 1.5
mg lipid phosphorus x 25 per 100 mg dry rod outer segments	31.0 ± 0.9	14.9 ± 0.5	16.0 ± 0.8

* Choline plasmalogen was not detected.

** Includes lysophosphatidyl ethanolamine.

*** Percentages in parentheses are the weighted sum of the values in Columns B and C.

3.3.3. *Phospholipid analysis of hexane extracted rod preparations*

Not all phospholipids present in cattle rod outer segments seem to be intimately associated with rhodopsin because a considerable fraction of the phospholipids could be removed with n-hexane without affecting the characteristic absorption peak at 500 nm and the photolytic properties.

Weight and lipid phosphorus determinations showed that 49°/o of total lipids and 51°/o of the phospholipids originally present were removed by hexane extraction. During the extraction the specific absorbance at 500 nm of the rhodopsin preparation increased by a factor of 1.27. From the amount of lipid removed an increase in specific absorbance by a factor of 1.24 was calculated. Since these two factors are equal within the limits of experimental error, it follows that the spectral integrity of rhodopsin was not affected by the hexane extractions.

The quantitative phospholipid composition of the hexane extract and of the hexane extracted residue is given in columns B and C of Table III. Combination of the data in these columns shows a fair agreement with the total phospholipid composition of the native material before hexane extraction (Column A, figures in parentheses).

The appearance after illumination of considerable amounts of extractable phospholipids, previously not extractable, has been reported by Ishimoto and Wald (1946), Adams (1967), and Poincelot and Abrahamson (1970a). This had been related to the possible existence of a phospholipid-retinaldehyde linkage in rhodopsin. We found that the increase in hexane-extractable material after exhaustive illumination of hexane-extracted rod outer segments in aqueous suspension was relatively small, 0.26 ± 0.07°/o of dry weight. Similar experiments with chloroform-methanol extraction from dark adapted and illuminated fresh rod outer segments showed no measurable difference between the lipid contents of the two extracts.

3.4. DISCUSSION

Table I shows that cattle rod outer segments contain 39°/o lipids, while the phospholipid content was found to be 31°/o. A comparison of our lipid analysis with that of other investigators is given in Table IV. Notable are the high lipid and phospholipid contents found by Fleischer and McConnell (1966) and Nielsen, Fleischer and McConnel (1970). These investigators stated that their high values may be caused by their isolation procedure, which involves vigorous homogenization resulting in preferential loss of protein. The fairly high phospholipid content reported by Poincelot and Zull (1969) may be ascribed to the fact that they equate the total lipid fraction with the

TABLE IV

LIPID COMPOSITION OF ROD OUTER SEGMENTS: RESULTS OF VARIOUS INVESTIGATIONS

	Cattle								Frog
	Percent of dry rod outer segments								
	Our results	Ref I	Ref II	Ref III	Ref IV	Ref V	Ref VI	Ref VII	Ref VIII
Total lipid	39		38 8	60	38 9			48	40 6
Cholesterol	3 1		0 9	3					1 7
Phospholipid	31 5	28	31 5	53		38 2	28	36	26 6
	Percent of lipid phosphorus								
Phosphatidyl ethanolamine *	39 2		51 0**			38 5	39 0	43 0	25 2
Phosphatidyl choline	34 7		31 0			51 7	41 2	39 8	49 4
Phosphatidyl serine	11 2		11 7***			7 2	12 9	13 7	9 5
Other phospholipids	11 3		6 3			2 6	6 7	3 5	15 9

* Including ethanolamine plasmalogen

** Originally listed as cephalin A

*** Originally listed as cephalin B

Ref I Collins et al, 1952
Ref II Sjostrand, 1959
Ref III Fleischer & McConnel, 1966
Ref IV Adams, 1967
Ref V Poincelot and Zull, 1969
Ref VI Anderson & Maude, 1970
Ref VII Nielsen et al, 1970
Ref VIII Lichberg & Hess, 1967

phospholipid fraction neglecting the other lipids (7 5%o in our determinations)

Table I indicates that the sum of phospholipids, cholesterol and retinaldehyde together account for only 35%o of the dry weight of rod outer segments while a total lipid content of 39%o was found The difference of 4%o might consist of glycolipids, for which Eichberg and Hess (1967) gave a value of 9 5%o in frog rod outer segments

Our analysis of the phospholipids present in the rod outer segments (Table III) indicates that the major phospholipid components are phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl serine This result is in agreement with those of other investigators (Table IV) Previously data for the phospholipid composition of cattle rod outer segments have been given by Sjostrand (1959) and by Poincelot and Zull (1969), (Table IV) In view of the primitive state of phospholipid analysis at that time, Sjostrand (1959) could only determine lecithin cephalin A (phosphatidyl ethanolamine), cephalin B (phosphatidyl serine) and sphingomyelin, which probably explains his high values for phosphatidyl ethanolamine and sphingomyelin The high phosphatidyl choline content (51%o) found by Poincelot and Zull (1969) may be due to the fact that it was obtained by a choline determination in the unfractionated lipid extract Results similar to ours have been reported very recently by Nielsen, Fleischer and McConnel (1970) and Anderson and Maude (1970) The analyses for cattle rod outer segments and those for frog outer segments (Eichberg and Hess, 1967) show general agreement in lipid pattern for these two species, except that a higher phosphatidyl choline and a lower phosphatidyl ethanolamine content was found in frog rod outer segments.

Table II shows that the rod outer segment lipids contain a large amount (34%o) of docosahexaenoic ($C_{22\ 6}$) fatty acid The presence of this fatty acid in large quantities in cattle rod outer segments has been confirmed by Poincelot and Abrahamson (1970b) (23%o), Nielsen et al (1970) (37%o), as well as by Anderson and Maude (1970) who showed that in phosphatidyl serine, phosphatidyl choline and phosphatidyl ethanolamine the $C_{22\ 6}$ fatty acid accounted for resp 32%o, 21%o and 32%o of the total fatty acid present in each of these phospholipids The high content of polyunsaturated fatty acid (approx. 50%o total, Table II) as well as the low values for cholesterol and sphingomyelin classify the rod outer segment membranes with the

metabolically active, fluid membrane typical of mitochondria (Fleischer and Rouser, 1965), rather than with the metabolically inactive, rigid myelin-type membranes which posess high cholesterol and sphingolipid contents and a low percentage of polyunsaturated fatty acids (Fleischer and Rouser, 1965, O'Brien, 1965)

The appearance after illumination of considerable amounts of extractable phospholipid (qualitatively detected), previously not extractable, has been claimed by Ishimoto and Wald (1947) and Adams (1967) We could not confirm these findings In the chloroform-methanol extraction as used by Adams (1967) we found no difference between illuminated and non-illuminated rod outer segments, in agreement with Poincelot and Zull (1969) This was to be expected, because chloroform-methanol treatment is generally considered to result in complete extraction of all phospholipids (except polyphosphoinositides) and in addition it bleaches rhodopsin In the case of hexane extraction we found a small effect, slightly less than 1 mole of phospholipid solubilized per mole retinaldehyde present This corresponds with approx $1^{o}/o$ of the total amount of phospholipids present in the rods In a more recent paper Poincelot and Abrahamson (1970a) reported that the amount of phospholipids that can be extracted from dark adapted rod outer segments with hexane is only $26^{o}/o$ of the total amount of phospholipids present, while illumination enhanced this percentage to a value of $58^{o}/o$ This $58^{o}/o$ lies close to the $51^{o}/o$ that could be extracted in the dark in our experiments with hexane Comparison of the hexane extraction procedures showed that our method of extraction (3 x 1 hour, room temperature) is probably more exhaustive than that used by Poincelot and Abrahamson (3 x 2 min, $4^{o}C$) It seems therefore to us that the large effect of light observed by Poincelot and Abrahamson (1970a) is rather that of an increase in rate of extraction with hexane than that illumination would make certain phospholipids more accessible to hexane, as suggested by these investigators

The main purpose of this analytical study of the phospholipids in rod outer segments was to obtain more knowledge about a possible linkage between phospholipids and retinaldehyde in rhodopsin Existence of an aldimine linkage between an amino group containing phospholipid and retinaldehyde in intact cattle rhodopsin (Poincelot et al, 1970) would require a molar ratio amino-phospholipid/retinaldehyde of at least one in native rod outer segments and also in hexane extracted rod outer segments, since the latter

have retained their photolytic properties. From the retinaldehyde content of the rod outer segments and the values of Table III we determined molar ratios of phosphatidyl ethanolamine and phosphatidyl serine to retinaldehyde of 30 and 10 for native rod outer segments and of 10 and 5 for hexane-extracted rod outer segments. Hence, enough phosphatidyl ethanolamine and phosphatidyl serine is present even in hexane extracted rod outer segments, to account for a possible aldimine link with retinaldehyde. Experiments reported by Krinsky (1958) and by Heller (1968a) indicated however that rhodopsin preparations having lower phospholipid contents than hexane extracted rod outer segments still show the characteristic properties of rhodopsin. This suggests that not all phospholipids present in hexane-extracted rod outer segments are essential for the photolysis of rhodopsin. We tried therefore in a further study to remove a larger part of the phospholipids from the outer segments without destroying the spectral integrity of rhodopsin. Such removal of phospholipids from rod outer segments by means of phospholipase C is described in Chapter 4.

3.5. SUMMARY

Cattle rod outer segments, isolated by means of a continuous sucrose gradient technique, contained 39°/o total lipid and 31.5°/o phospholipid on a dry weight basis. The fatty acid composition of the total rod outer segment lipids was determined by means of gas-liquid chromatography. The three predominant fatty acids are : palmitic acid (19.4°/o w/w of total fatty acids), stearic acid (23.1°/o) and docosahexaenoic acid (34.3°/o). Quantitative analysis of the phospholipids by two-dimensional thin-layer chromatography showed that the three major phospholipids are: phosphatidyl ethanolamine (35.4°/o), phosphatidyl choline (34.7°/o) and phosphatidyl serine (11.2°/o).

Half of the total amount of phospholipids was extractable with n-hexane without changing the spectral and photolytic properties of rhodopsin. Relatively more phosphatidyl ethanolamine than phosphatidyl choline was extracted by hexane. The molar ratio of phosphatidyl ethanolamine to retinaldehyde was reduced from 30 to 10 by hexane extraction.

CHAPTER 4

REMOVAL OF PHOSPHOLIPIDS FROM THE ROD OUTER SEGMENT BY TREATMENT WITH PHOSPHOLIPASE C

4.1. INTRODUCTION

In Chapter 3 it was concluded that phosphatidyl ethanolamine is a major phospholipid in cattle rod outer segments. The quantity of this phospholipid was, even after hexane extraction of the outer segments, amply sufficient to account for the existence of an aldimine linkage with retinaldehyde in rhodopsin as suggested by Poincelot et al (1970). As discussed in 1.4 the presence of such a linkage in rhodopsin is doubted by other investigators but their evidence was indirect and did not appear to be conclusive to us. We attempted, therefore, to find a more direct approach to establish whether phosphatidyl ethanolamine can play a role in the binding of the chromophore in rhodopsin. Our approach consisted of removal of the phospholipids from the rhodopsin complex as completely as possible without affecting the spectral integrity of the visual pigment. For this purpose phospholipase C treatment was chosen as previously utilized by Krinsky (1958). In stead of the Clostridium perfringens enzyme employed by Krinsky, the phospholipase C from Bacillus cereus was used, since its broad spectrum of substrate specificity (Van Deenen and De Haas, 1966) offered hope of removing more phospholipids.

This chapter describes the results of this study, demonstrating that at least 95% of the phospholipids in the rod outer segments are non-essential for the maintenance of the spectral and photolytic properties of rhodopsin and that in enzymatically lipid-denuded preparations, having a lipid phosphorus to retinaldehyde molar ratio of only 4, the lipid ethanolamine content is only 0.2 mole per mole of retinaldehyde. This ratio is definitely too low to account for the presence of the retinaldehyde as retinylidene phosphatidyl ethanolamine in spectrally intact rhodopsin. Parts of this chapter have been published previously (Borggreven et al, 1971).

4.2. MATERIALS AND METHODS

4.2.1. *Isolation of rod outer segments*

Cattle rod outer segment membranes were isolated by means of a continuous sucrose gradient technique as previously described (2.2.1). When the preparations were used for treatment with phospholipase C, the final two washings with water in the original procedure (2.2.1) were replaced by two washings with the TRIS-maleate incubation buffer (see under 4.2.3.). Normally the preparations were not lyophilized prior to incubation. These rod outer segment membrane preparations will be further designated as rod preparations.

4.2.2. *Preparation of phospholipase C from Bacillus cereus*

Phospholipase C (E.C. 3.1.4.3) was obtained from cultures of B.cereus (NCTC 6349) by a modification of the method of Ottolenghi (1965). Ten ml of an overnight culture in brain-heart infusion were added to 2 l of a culture medium containing 6.0 g trypton, 7.2 g NaCl, 9.0 g $Na_2HPO_4.2\ H_2O$ and 7.2 g glucose in water. The culture was incubated for 6 hr at 37° under aeration, after which the temperature was gradually raised to 50° in the course of 1 hour and was maintained at 50° for another hour to destroy a hemolysin produced by this micro-organism (Ottolenghi, 1965). The bacteria were then sedimented by centrifugation for 20 min at 12,800 x g and $(NH_4)_2SO_4$ was added to the supernatant solution to 75°/o saturation. During this procedure the pH was kept at 7.2 by the addition of 2 N NaOH. The resulting precipitate was sedimented by centrifugation for 20 min at 12,800 x g. The supernatant solution was discarded and the sediment dissolved in 50 ml of 0.2 M TRIS-maleate buffer (pH 7.2). This solution was dialyzed against 3 l of the same buffer for 3 one-hour periods. The dialyzed solution, stored at 4°, remained active for at least three months.

4.2.3. *Incubation with phospholipase C*

For incubation with the B. cereus enzyme, fresh non-lyophilized rod preparations isolated from 50-70 retinas were homogenized in a Potter-Elvehjem homogenizer with 0.8 ml of the dialyzed enzyme solution and 2.2 ml of 0.2 M TRIS-maleate buffer (pH 7.2) and shaken for 4 hours at 37° under N_2. The suspension was centrifuged for 10 min at 18,100 x g and when desired the sediment was again incubated under the same conditions with the same amount of fresh enzyme and buffer for another hour. After centrifugation for 10 min at 18,100 x g the sediment was twice washed with distilled water and was lyophilized. All manipulations were carried out in dim red light or in the dark.

Phospholipase C from Cl. welchii was obtained from Calbiochem (Los Angeles, Cal., U.S.A.). Incubation with Cl. welchii enzyme was performed in essentially the same manner as incubation with phospholipase C from B. cereus.

4.2.4. *Lipid extractions*

For complete lipid extraction a weighed amount of phospholipase C treated or phospholipase C treated-hexane extracted rod preparation was extracted three times for one hour with 0.05 - 0.1 ml of a $CHCl_3$ - CH_3OH - H_2O mixture (60:30:3:6, by vol.) per mg preparation as described under 3.2.2. Unless otherwise indicated the total lipid extract was washed with a solution of 0.1 M KCl according to Folch et al (1957).

Hexane extractions of phospholipase C treated or native rod preparations were performed under dim red light and under nitrogen. The preparations (50 - 75 mg dry weight) were extracted three times for one hour with a total of 9 ml of n-hexane as described above for the chloroform-methanol extractions.

In either case, the combined lipid extract was evaporated to near-dryness and stored in a known volume of benzene-ethanol (4:1, by vol) at -20° under N_2.

From the extracted preparations the remaining solvent was removed by a gentle stream of nitrogen followed by drying in vacuo. The preparations were stored (the hexane extracted preparations in darkness) at -20° under N_2.

4.2.5. *Lipid analysis*

Lipid phosphorus was determined in aliquots of the washed lipid extract, containing 0.5 - 2.5 μg phosphorus, by means of a modified Fiske-Subbarow method (see 3.2.6) after digestion in H_2SO_4 - $HClO_4$ (all volumes scaled down 4 times).

Phospholipid analyses were carried out by phosphorus determinations in the spots obtained after two-dimensional thin-layer chromatography on silicagel (3.2.6). For each analysis an amount of lipid extract containing 10-15 μg lipid-P was used.

Ethanolamine and serine analyses in phospholipase C treated-hexane extracted rod preparations were carried out by means of an automatic amino acid analyzer. A known aliquot of the washed or unwashed lipid extract, after evaporation of solvent, was hydrolyzed with 6 N HCl in sealed, evacuated tubes for 3 hours as described by Poincelot and Abrahamson (1970a). When analyses were performed directly on the rod outer segment preparations without previous lipid extraction, hydrolysis was continued for 22 hours in order to avoid interference by residual peptides. After removal of the hydrochloric acid by evaporation the sample was applied to the column of a Technicon amino acid (column filling: chromobeads A, 21 μ, Technicon instruments Corp., Chertsey, England). The one-column principle developed by Piez and Morris (1960) was applied with elution by a continuous gradient of methanol in sodium citrate-hydrochloric acid buffer (Na^+ : 0.186 $\longrightarrow$ 0.85 M; pH: 2.86 $\longrightarrow$ 5.00; CH_3OH : 6.6 $\longrightarrow$ 0 vol. %) at a column temperature of 60°.

Control experiments in which pure phosphatidyl ethanolamine and phosphatidyl serine were subjected to this procedure, yielded quantitative recovery of ethanolamine and serine.

4.2.6. *Mild hydrolysis of phospholipids*

Phospholipids were deacylated under mild alkaline conditions as described by Dawson (1960). The resulting phosphate esters were identified by paper electrophoresis carried out for 2 hours at 30 - 40 V/cm in a pyridine-acetic acid-water buffer (1:10:89, by vol.; pH 3.5) (Dawson et al, 1962), against reference compounds obtained by deacylation of reference phospholipids.

4.2.7. *Spectral measurements*

In order to determine rhodopsin, dry rod preparations (native, phospholipase C treated, or phospholipase C treated-hexane extracted) were homogenized in 67 mM phosphate buffer pH 6.8 containing 1°/o digitonin or 1°/o Triton X-100 (about 1 mg preparation/ml). Digitonin was found to be a suitable extractant for native and phospholipase C treated rod preparations, but it extracted rhodopsin incompletely from phospholipase C treated-hexane extracted preparations even after shaking for 1 hour. Triton X-100 gave complete extraction from all three preparations in 10 min. After extraction the suspension was centrifuged for 5 min at 18,100 x g, and 10 μl of 1 M hydroxylamine (pH 6.8) was added to 200 μl of the supernatant solution.

For determination of rhodopsin during the phospholipase C incubation 50 μl of suspension were added to 1 ml of 1°/o Triton X-100 in 67 mM phosphate buffer (pH 6.8). The mixture was homogenized for 10 min, and treated like the extracted preparations described above.

The spectra were measured before and after exhaustive illumination by a 75 W tungsten lamp at a distance of 20 cm for 10 min through ultraviolet and infrared filters (KG-1 and GG-3 filters, thickness 3 mm each, Schott-Jena, Mainz, Germany). The difference in absorbance of a preparation at 500 nm before and after illumination is designated as ΔA_{500}. The ΔA_{500} for solutions containing 1 mg of dry rod outer segment preparation per ml solution is indicated as ΔA_{500} per mg. From the latter value and the molar absorbance of rhodopsin, the amount of rhodopsin present was calculated on a molar basis.

4.2.8. *Determination of the molar absorbance coefficient of rhodopsin*

The molar absorbance coefficient at 500 nm of phospholipase C treated and phospholipase C treated and phospholipase C treated-hexane extracted rhodopsin was determined in the same manner as described in Chapter 2 for native rhodopsin.

4.3.1. *Incubation with phospholipase C from various sources*

Phospholipase C splits glycerophospholipids into hydrophobic diglycerides and hydrophilic phosphate esters (Fig. 11). Treatment of rod suspensions with the enzyme results in release of phosphate esters into the aqueous medium, leaving a residue with decreased phosphorus content, but still containing the diglycerides. The latter can be extracted with hexane.

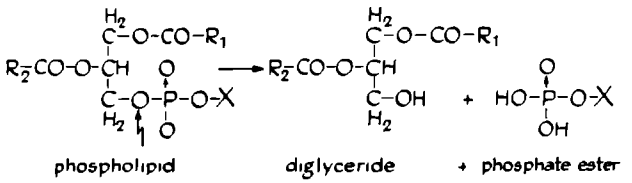


Figure 11. Action of phospholipase C on a phospholipid.
X represents the polar end-group of the phospholipid.

In view of the different substrate specificities of phospholipase C preparations from various sources (Van Deenen and De Haas, 1966), we investigated the action of phospholipase C obtained from Cl.welchii and that from B.cereus on hexane extracted rod membranes. Phospholipase C from B. cereus is more effective in hydrolyzing rod phospholipids than the Cl. welchii enzyme (Fig.12) and also than the Cl. perfringens enzyme employed by Krinsky (1958), which removed only 80°/o of the phospholipids present in native rhodopsin. Since our purpose was to obtain rod preparations with minimal phospholipid content, we used the B. cereus enzyme for all further incubations.

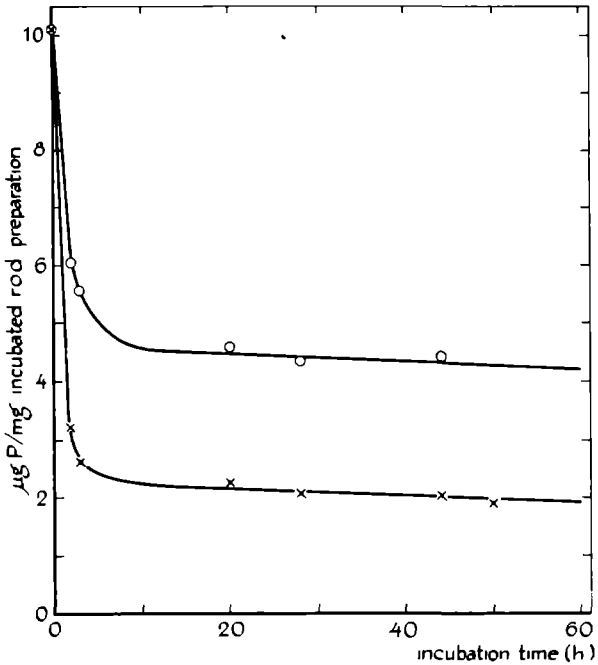


Figure 12. Phosphorus content of lyophilized, hexane-extracted rod preparations upon treatment with phospholipase C from Cl. welchii (o—o—o) or from B. cereus (x—x—x).
After incubation with phospholipase C the preparations were washed twice with distilled water and then lyophilized. Phosphorus was determined in the dry preparations.

4.3.2. *Incubation with phospholipase C from B. cereus and subsequent hexane extraction*

Spectral measurements

In order to determine whether rhodopsin remains spectrally intact during phospholipase C incubation, aliquots were taken from the incubation suspension at various times and the rhodopsin content was determined as shown in Fig.13, curves B and C. Data in curve C are for incubations in which

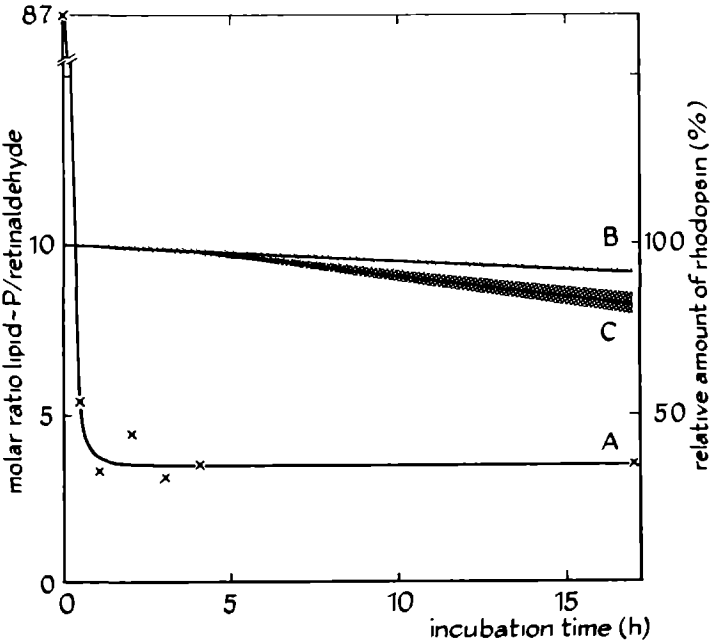


Figure 13. Amounts of lipid phosphorus and spectrally intact rhodopsin in rod preparations upon treatment with phospholipase C.
Curve A represents the amount of lipid phosphorus per mole of rhodopsin chromophore upon incubation Curves B and C show the relative amounts of intact rhodopsin present at different times of incubation in aliquots of the incubation suspension. Data in curve C are for incubations in which enzyme and buffer were refreshed after the initial 4-hr incubation period. The shaded areas around the curves represent the standard error of the slopes. The amount of rhodopsin at the start of the incubation was taken as 100%.

after the initial 4 hr-incubation period enzyme and buffer were refreshed. Curves B and C represent results for 4 and 6 preparations, respectively. Although the slopes of B and C are significantly different from zero (Student test. $t_{20} = 2.1$, $P = 0.05$ for B and $t_{20} = 5.2$, $P < 0.001$ for C), the corresponding loss of rhodopsin after the 4 plus 1 hr incubation period is only 3.6% (S.E. 1.0%) of the amount of rhodopsin present at the start of the incubation. So we may conclude that rhodopsin remains spectrally almost

TABLE V

CHARACTERISTICS OF CATTLE ROD OUTER SEGMENT PREPARATIONS UPON INCUBATION WITH PHOSPHOLIPASE C AND SUBSEQUENT HEXANE EXTRACTION

Values are given with their standard errors and between parentheses the number of independent determinations.

	Native	Pl-ase C treated	Pl-ase C treated hexane extracted
Molar absorbance at 500 nm	43,000 ± 700 (23)	41,700 ± 1600 (12)	41,700 ± 1350 (12)
Calculated weight	≡ 100°/o	93 *	63 **
Calculated ΔA_{500}/mg***	≡ 100°/o	108	159
Experimental ΔA_{500}/mg	≡ 100°/o	103 ± 3.1 (8)	149 ± 7.4 (7)

* Yield after phospholipase C incubation if loss of weight is caused only by removal of water-soluble phosphate esters from the phospholipids.

** Yield after phospholipase C incubation and subsequent hexane extraction if loss of weight is caused only by removal of the diglyceride moieties of the hydrolyzed phospholipids and other loosely bound, non-hydrolyzed lipids

*** Values expected from the calculated yields.

entirely intact during the enzymatic treatment. In agreement with this observation, the molar absorbance coefficient of rhodopsin at 500 nm, calculated from its ΔA_{500} and its retinaldehyde content, was not significantly altered upon phospholipase C treatment (Table V).

Similarly, hexane extraction after enzymatic incubation, influenced neither the molar absorbance coefficient (Table V) nor the shape of the absorption spectrum before and after illumination (Fig.14). Strictly speaking,

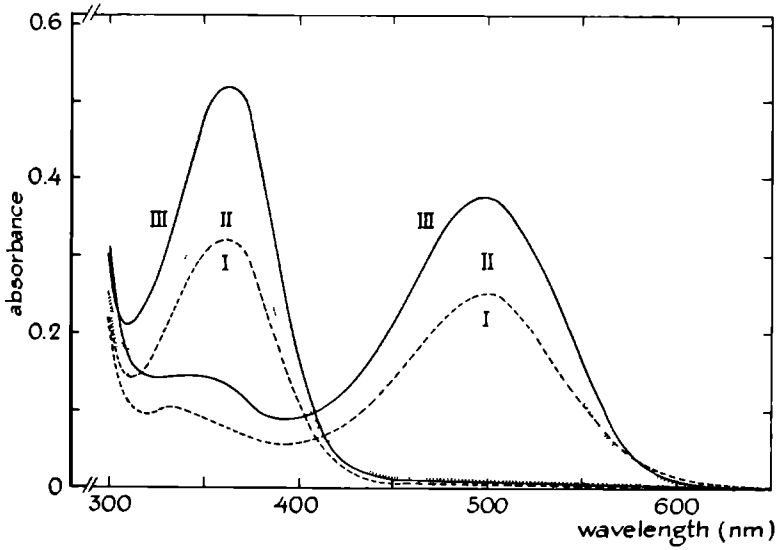


Figure 14. Spectra of native (I), phospholipase C treated (II) and phospholipase C treated-hexane extracted (III) rod preparations before (I, II and III) and after (I', II' and III') exhaustive illumination.
The spectra were measured in a solution of 1°/o Triton X-100 in 67 mM phosphate buffer pH 6.8 to which hydroxylamine was added to a final concentration of 48 mM.

the constancy of the molar absorbance coefficient does not guarantee that all rhodopsin remains intact during hexane extraction. The fact that the absorp-

tion spectra of native rhodopsin, phospholipase C treated and phospholipase C treated-hexane extracted rhodopsin have the same shape (Fig.14) does not prove this point either. We can, however, argue that the rhodopsin remains intact during these two treatments from the increase observed in the ΔA_{500} per mg preparation (Table V, columns 3 and 4). Calculations based on the phospholipid content of untreated rods (Chapter 3) and after enzyme treatment and hexane extraction, respectively, indicate that the ΔA_{500} per mg (= 100°/o for native rod preparations) should rise to 108°/o after enzyme incubation and to 159°/o after subsequent hexane extraction due to the removal of phosphate esters in the first stage and of diglycerides and loosely bound lipids in the second stage. The close agreement between the calculated and experimentally found values (Table V) and the intactness of rhodopsin during enzyme incubation (5hr at 37°) strongly suggest that rhodopsin remains spectrally intact also during the hexane extraction procedure. There is still the remote possibility that breakdown of rhodopsin would be masked by the solubilization of non-lipid material during hexane extraction, but this is unlikely in view of the fact that the observed yield on a weight basis was not significantly different from the yield calculated on the basis of lipid removal during hexane extraction (observed yield 91°/o of the calculated yield, S.E. 4°/o, n = 5, P = 0.09).

Lipid-phosphorus determinations

A rapid decrease in the phospholipid content of the preparation was observed upon incubation with phospholipase C from B. cereus, when lipid-phosphorus was determined in aliquots of the washed chloroform-methanol extracts of known amounts of lyophilized preparations (Fig. 13). Within 4 hr the original level of 87 moles of lipid-phosphorus per mole of rhodopsin chromophore had fallen to 3.5 (Table VI), which did not decrease further during an additional 13 hr of incubation (Fig. 13). Reincubation with fresh enzyme-buffer medium for up to 4 hr after an initial 4-hr incubation period did not further lower the lipid-phosphorus content of the preparations. A single incubation period of 4 hr is therefore sufficient to remove the maximal amount of phospholipids. This indicates that the constant lipid-phosphorus level after the initial incubation period was not due to enzyme denaturation or inhibition of the enzyme by the water soluble products of hydrolysis.

TABLE VI

LIPID-PHOSPHORUS CONTENT OF NATIVE, PHOSPHOLIPASE C TREATED, AND PHOSPHOLIPASE C TREATED-HEXANE EXTRACTED CATTLE ROD OUTER SEGMENT PREPARATIONS

Rod outer segment preparation	$\frac{\mu g \text{ lipid-P}}{\text{mg rod prepn.}}$	$\frac{\text{lipid-P}^*}{\text{retinaldehyde}}$
Native	12.6	87
Pl-ase C treated	0.72 ± 0.06 (5)	3.5 ± 0.39 (4)
Pl-ase C treated-hexane extracted	1.17 ± 0.06 (5)	4.4 ± 0.34 (4)

* Molar ratio; the retinaldehyde content was calculated from ΔA_{500}. In parentheses the number of preparations analyzed.

Extraction of phospholipase C treated preparations with hexane did not remove significant amounts of lipid-phosphorus as indicated by the data in Table VI and by analysis of the hexane extract, in which less than 0.1 mole phosphorus was found per mole of retinaldehyde present in the nonextracted material.

4.3.3. *Lipid analysis of phospholipase C treated-hexane extracted rod preparations*

An analysis of the phospholipids present in phospholipase C treated rod preparations was difficult, since the large excess of diglycerides disturbed the thin-layer chromatography of the relatively few phospholipids remaining after enzyme treatment. Removal of the diglycerides without extraction of phospholipids could be achieved by hexane treatment. Qualitative analysis of the

hexane extract by thin-layer chromatography according to Storry and Tuckley (1967) indeed showed the major spot to represent the diglycerides, while cholesterol and monoglycerides as well as tri-glycerides or cholesterol esters were found in relatively small amounts.

Quantitative analysis of the lipid extract of phospholipase C treated-hexane extracted rod preparations by means of two-dimensional thin-layer chromatography (Fig.15) showed that the major phospholipids in these rod

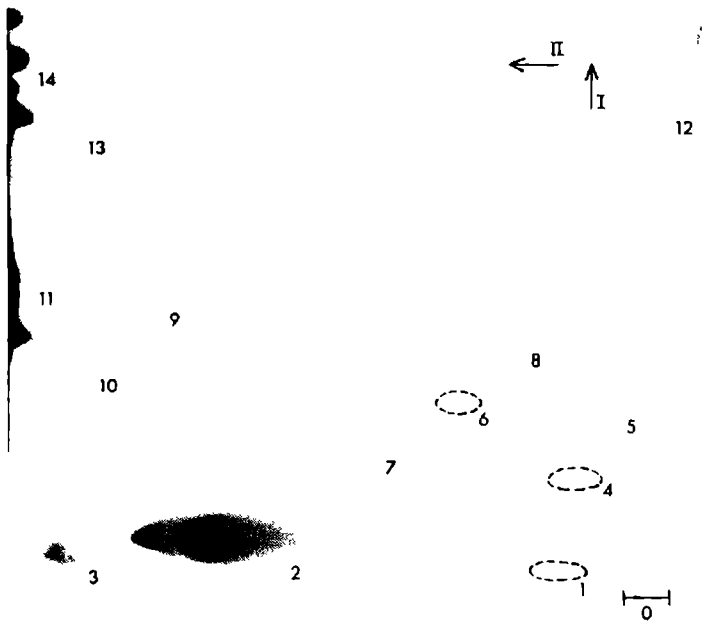


Figure 15. Two dimensional thin-layer chromatogram of a total lipid extract of phospholipase C treated-hexane extracted cattle rod outer segments.
Development in the first dimension with chloroform-methanol-7N ammonia (90:54:11, by vol.), in the second dimension : chloroform - methanol - acetic acid - water (90:40:12:2, by vol.); staining with iodine vapour. Identity of the spots: O, origin; 1, lysophosphatidyl serine; 2, phosphatidyl serine; 3, phosphatidic acid; 4, lysophosphatidyl choline; 5, sphingomyelin; 6, lysophosphatidyl ethanolamine; 7, phosphatidyl inositol; 8, phosphatidyl choline; 9, phosphatidyl ethanolamine; 11, diphosphatidyl glycerol; 14, retinaldehyde plus other neutral lipids; 10, 12 and 13, unknown lipids.

preparations are phosphatidyl serine, diphosphatidyl glycerol and sphingomyelin. The quantitative results are listed in Table VII. The identity of the spots on the thin-layer chromatograms were confirmed by specific staining

TABLE VII

PHOSPHOLIPID COMPOSITION OF PHOSPHOLIPASE C TREATED-HEXANE EXTRACTED CATTLE ROD OUTER SEGMENT MEMBRANES

The values with their standard errors are averages for 4 preparations.

	% of total lipid-P
Phosphatidyl serine	36.8 ± 4.7
Diphosphatidyl glycerol	17.2 ± 3.3
Sphingomyelin	13.2 ± 1.1
Phosphatidyl ethanolamine	2.4 ± 1.5
Lysophosphatidyl ethanolamine	2.0 ± 1.1
Phosphatidyl choline	3.1 ± 1.2
Lysophosphatidyl choline	1.2 ± 0.5
Lysophosphatidyl serine	3.9 ± 1.7
Phosphatidic acid	2.9 ± 0.7
Others	7.3 ± 0.5
Recovery	90.0 ± 3.8

reactions (Skidmore and Entenman, 1962; Dittmer and Lester, 1964; Bishel and Austin, 1963) and by comparison with natural or synthetic reference compounds as described in 3.2.3. The identity of phosphatidyl serine was further checked by means of amino acid analysis after isolation from the chromatoplate. This yielded clear evidence that the nitrogenous moiety of the phospholipid consisted of serine. The presence of phosphatidyl serine and diphosphatidyl glycerol as major phospholipids was further confirmed by the demonstration of glyceryl phosphoryl serine and bis (glyceryl phosphoryl)

TABLE VIII

ETHANOLAMINE AND SERINE CONTENT OF NATIVE AND PHOSPHOLIPASE C TREATED-HEXANE EXTRACTED CATTLE ROD OUTER SEGMENT PREPARATIONS*

	Molar ratios			
Rod outer segment preparation	Ethanolamine/retinaldehyde**			Serine/retinaldehyde**
	Washed lipid extract	Unwashed lipid extract	Total preparation	Washed lipid extract
Native	30 ****			9 ****
Pl-ase C treated hexane extracted				
AAA ***	0.24 ± 0.06 (3)	0.30 ± 0.09 (2)	0.69 ± 0.05 (4)	1.9 ± 0.37 (3)
TLC ****	0.20 ± 0.03 (4)			1.8 ± 0.26 (4)

* Values are given with their standard errors and in parentheses the number of rod preparations analyzed.

** The retinaldehyde content was calculated from ΔA_{500}.

*** Determined by quantitative analyses on the amino acid analyzer (AAA).

**** Determined by quantitative two-dimensional thin-layer chromatography on silica gel (TLC).

glycerol in the alkaline hydrolysate of the lipid extracts by the method of Dawson (4.2.6). Minor phospholipids were not detected in this manner because of the very low content of phospholipids in the rod preparations (1.17 μg total lipid-P/mg).

We frequently observed that the sphingomyelin spot was separated into two adjacent spots. Such a separation of the sphingomyelin spot on thin layers of silicagel has been reported previously (Wood and Holton, 1964) and shown to be the result of differences in fatty acid composition. Since we could not find any difference in specific staining reactions between the two spots on our chromatograms we assumed that both spots contained sphingomyelin. The double spot of phosphatidyl serine may be the result of the presence of salts of this phospholipid with different cations (Broekhuyse, 1968). Also in this case we could not find any difference in staining between head and tail of the spot. It was therefore assumed that phosphatidyl serine was the only compound present. This assumption is supported by the good agreement between the determinations of lipid serine by thin-layer chromatography and by amino acid analysis (Table VIII, column 4).

Using the data of Table VII, we were able to compare the molar ratio's between individual phospholipids and the rhodopsin chromophore in phospholipase C treated-hexane extracted rod preparations. These ratio's for several phospholipids, compared to those in native rhodopsin, are shown in Fig.16. Phosphatidyl ethanolamine and phosphatidyl choline appeared to be hydrolyzed almost completely by phospholipase C, whereas sphingomyelin and diphosphatidyl glycerol remained intact. About 20^{o}/o of the phosphatidyl serine originally present was resistant against enzymatic attack.

In view of the possible existence of an aldimine link between the rhodopsin chromophore and an amino group-containing phospholipid, the molar ratio's, lipid ethanolamine/retinaldehyde and lipid serine/retinaldehyde, were calculated from the thin-layer chromatographic analyses (Table VII) as well as from ethanolamine and serine determinations by means of the amino acid analyzer. Both techniques revealed that only 0.2 mole of ethanolamine-phosphoglycerides (lysophosphatidyl ethanolamine + phosphatidyl ethanolamine) per mole retinaldehyde was present in the KCl-washed lipid extracts of the preparations (Table VIII). To exclude any ambiguity, additional determinations were carried out in the unwashed chloroform-methanol extract and in the non-extracted preparation. The values in Table VIII indicate that there

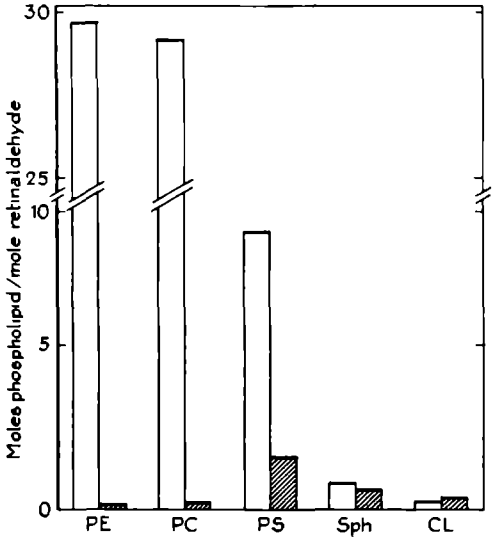


Figure 16. Amounts of various phospholipids present in native (open bars) and phospholipase C treated, hexane extracted (hatched bars) rod preparations. PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PS = phosphatidyl serine, Sph = sphingomyelin, CL = cardiolipin (diphosphatidyl glycerol)

was no significant loss of ethanolamine due to the KCl-washing procedure, but there was a significantly higher ethanolamine/retinaldehyde ratio in the material prior to extraction by chloroform-methanol. However, even this ratio (0.69 ± 0.05) was significantly less than one. The higher ratio of 0.69 as compared to that for the chloroform-methanol extract means that some ethanolamine remains in the residue after chloroform-methanol extraction. Qualitative analysis by means of the amino acid analyzer showed indeed the presence of ethanolamine in this residue. Whether this residual ethanolamine represents lipid-ethanolamine not extractable with chloroform-methanol or ethanolamine (or its phosphate) strongly bound to protein cannot be determined from our experiments. The first possibility seems unlikely because of the known effectiveness of chloroform-methanol as a lipid extractant.

The enzyme incubation experiments were initially performed with lyophilized rod preparations, previously extracted with hexane, since this solvent extracts half of the phospholipids from rod preparations without affecting the spectral integrity of rhodopsin (see Chapter 3). However, enzymatic hydrolysis in rod preparations, not previously extracted with hexane, appeared to occur faster and to about the same degree (compare Figs.12 and 13). Moreover, in omitting the initial hexane extraction lyophilization became superfluous and hence fresh rod preparations could be used. Omission of lyophilization reduced the level of lysophosphatidyl ethanolamine, which probably originates from phosphatidyl ethanolamine during this procedure (Fleischer and Rouser, 1965). This was particularly important in view of our observation that this lyso compound was not readily hydrolyzed by the enzyme, thus giving rise to higher residual lipid ethanolamine levels.

Table II shows that treatment with phospholipase C removes 95°/o of the lipid phosphorus from native rod preparations. Subsequent hexane extraction solubilizes the diglyceride part of the hydrolyzed phospholipids. It was demonstrated in Table V and Fig.13 that the characteristic spectral properties of rhodopsin are retained in these treatments. This means that neither the polar moiety nor the diglyceride moiety of 95°/o of the phospholipids in the rod outer segment are essential for the spectral and photolytic integrity of rhodopsin.

Fig. 16 shows that diphosphatidyl glycerol and sphingomyelin are not hydrolyzed by phospholipase C when present in the rod outer segment membranes, although hydrolysis of these substances by this enzyme has been reported (Slein and Logan, 1965; De Haas et al, 1966). Only about 80°/o of the phosphatidyl serine was hydrolized by phospholipase C, while phosphatidyl ethanolamine and phosphatidyl choline were virtually completely hydrolyzed. The amount of residual phosphatidylserine (1.6 mole/mole chromophoric retinaldehyde) exceeds the minimal amount of 1 mole phosphatidyl serine required for a phosphatidyl serine-retinaldehyde aldimine link. Thus too much phosphatidyl serine is present in the phospholipase C treated preparations to draw conclusions about the presence or absence of such a link in rhodopsin.

The residual phosphatidyl serine in the treated rod preparations became

susceptible to hydrolysis by phospholipase C after it had been extracted from the rod preparation by a chloroform-methanol mixture This indicates that this phosphatidyl serine may be protected against enzymatic attack by an association with rhodopsin This association could consist of an aldimine linkage between retinaldehyde and phosphatidyl serine The persistence of a significant amount of phosphatidyl serine after treatment with phospholipase C from B cereus is, however, not limited to rhodopsin Roelofsen (1968) observed that in erythrocyte ghosts treated with the same B cereus enzyme preparation only 70°/o of the phosphatidyl serine and 10°/o of sphingomyelin against 90-95°/o of the ethanolamine phosphoglycerides and phosphatidyl choline were hydrolyzed Hence, it is not necessary to assume that the incomplete hydrolysis of phosphatidyl serine is due to binding of the chromophore to this phospholipid In the next chapter this matter is settled

The amount of 0 2 mole lipid ethanolamine per mole of retinaldehyde listed in Table VIII is sufficiently below one to rule out the possibility that the retinaldehyde group in phospholipase C treated rhodopsin is linked to phosphatidyl ethanolamine Before applying this conclusion to native rhodopsin we must consider the possibility that during phospholipase C treatment there might occur a transiminization reaction shifting retinaldehyde from phosphatidyl ethanolamine in native rhodopsin to another amino group in opsin Since the spectrum of rhodopsin remained unaltered during enzyme treatment (Fig 14), this would imply the existence of two rhodopsins with different binding sites for the retinaldehyde but with identical spectral properties, and this appears extremely unlikely Hence, we conclude that in native cattle rhodopsin the retinaldehyde cannot be linked to phosphatidyl ethanolamine

This conclusion is at variance with that of Poincelot et al (1970) who deduced a link of retinaldehyde to phosphatidyl ethanolamine in native cattle rhodopsin from their ability to extract the chromophore nearly quantitatively from native rhodopsin preparations as N-retinylidene phosphatidyl ethanolamine This conclusion was initially supported by Akhtar and Hirtenstein (1969), who found that 70°/o of the chromophore was extractable with methanol from rhodopsin treated with trichloroacetic acid in the dark Thin-layer chromatography indicated that the retinaldehyde was largely present as N-retinylidene-phosphatidyl ethanolamine However, in a later paper (Hirtenstein and Akhtar, 1970) these authors have partly retracted their conclusions

because of lack of reproducibility of chromophore extraction by methanol for a number of different rhodopsin preparations They state that the only firmly established aspect of the chromophoric binding site remains the demonstration that in the metarhodopsin II stage the retinyl moiety is linked to an ϵ-amino group of lysine The discrepancy between our results and those of Poincelot et al (1970) is probably due to artefactual transiminization of the chromophore to phosphatidyl ethanolamine during their extraction with acidified methanol Recently, Daemen et al (1971) have shown that a $10^{-3.5}$ M HCl concentration in methanol, as used by Poincelot et al (1970), is insufficient to prevent transiminization in a model system containing retinylidene imine and phosphatidyl ethanolamine In addition, these studies showed that treatment of rhodopsin with methanol - $10^{-3.5}$ M HCl indeed yielded N-retinylidene phosphatidyl ethanolamine, but when the HCl concentration was raised to 10^{-2} M this compound was no longer extracted, although retinylidene phosphatidyl ethanolamine is freely soluble in strongly acidified methanol These findings are in accordance with our conclusion that in native cattle rhodopsin there does not exist an aldimine linkage between the chromophoric retinaldehyde and phosphatidyl ethanolamine

4 5 SUMMARY

Phospholipids present in cattle rod outer segments were hydrolyzed by phospholipase C from B cereus In this way, the molar ratio of lipid phosphorus to retinaldehyde bound in rhodopsin could be reduced from 87 to 4

During this treatment the spectral integrity of the rhodopsin was not affected as appeared from the shape of its absorption spectrum and the determination of the amount of rhodopsin from the value of ΔA_{500} before and after phospholipase C treatment Further evidence that the spectral integrity was not affected is supplied by the constancy of the molar absorbance of rhodopsin at 500 nm during enzyme treatment

Hexane extraction of the phospholipase C treated material did not further reduce the lipid phosphorus to retinaldehyde ratio, but did remove diglycerides arising from the hydrolyzed phospholipids The photolytic properties of phospholipase C treated rhodopsin also remained intact during the hexane extraction From this we conclude that neither the polar moiety nor

the diglyceride part of 95%/o of the phospholipids in the rod outer segments are essential for the spectral integrity of rhodopsin.

Quantitative analysis of the phospholipids present in the phospholipase C treated-hexane extracted preparations showed that they consisted of 1.6 mole phosphatidyl serine, 0.4 mole diphosphatidyl glycerol, 0.6 mole sphingomyeline, 0.1 mole phosphatidyl ethanolamine and 0.1 mole lysophosphatidyl ethanolamine per mole of retinaldehyde. These findings rule out the possibility that the retinaldehyde group in rhodopsin is linked to phosphatidyl ethanolamine or its lyso derivative.

CHAPTER 5

REMOVAL OF PHOSPHATIDYL SERINE FROM ROD OUTER SEGMENT PREPARATIONS BY PHOSPHOLIPASE A TREATMENT

5.1. INTRODUCTION

In Chapter 4 we showed that phospholipase C incubation and subsequent hexane extraction removed sufficient phosphatidyl ethanolamine from the rod preparations to exclude an aldimine linkage between retinaldehyde and phosphatidyl ethanolamine in rhodopsin. However, such a conclusion was not possible for phosphatidyl serine, since the molar ratio between retinaldehyde and phosphatidyl serine decreased only to 1.6 after phospholipase C treatment.

This chapter describes the removal of the major part of the phosphatidyl serine present in the phospholipase C treated-hexane extracted rod preparations by means of phospholipase A treatment followed by extraction with serum albumin. In this way the phosphatidyl serine level could be reduced sufficiently to conclude that an aldimine linkage between this phospholipid and retinaldehyde in rhodopsin cannot exist either.

5.2. METHODS AND MATERIALS

5.2.1. *Incubation with phospholipase A_2*

Phospholipase C treated-hexane extracted rod preparations (for preparation, see 4.2.3 and 4.2.4) were homogenized in a Potter-Elvehjem tissue grinder in 0.2 M Tris-maleic acid buffer (pH 7.2) containing 0.01 M $CaCl_2$(12 mg rod preparation / ml buffer). To the resulting suspension 1 mg lyophilized Crotalus adamanteus snake venom (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was added per 6 mg rod preparation. The mixture was then incubated with vigorous shaking for 1.5 hr at 37°C under nitrogen. After the incubation period the suspension was centrifuged for 30 min at 39,600 x g at 4°C.

For lipid analysis the pellet was washed and resedimented twice with distilled water at 4°C and then lyophilized.

For extraction with serum albumin, the pellet obtained after centrifugation of the incubation mixture was resuspended in 0.05 M glycylglycine buffer pH 7.40 and sedimented by centrifugation for 30 min at 39,600 x g at 4°C. The resulting sediment was then extracted with an aqueous solution of serum albumin as described in the next section. The phospholipase C treated-hexane extracted, phospholipase A treated rod outer segment preparations will be further designated as $R_{C,H,A}$.

5.2.2. *Extraction with serum albumin*

The glycylglycine washed phospholipase A incubated rod preparation (5.2.1) was suspended in a 1°/o solution of bovine serum albumin (Behringwerke A.G., Marburg-Lahn, Germany) in 0.05 M glycylglycine buffer pH 7.4 (about 2 mg rod preparation/ml serum albumin solution). The suspension was homogenized in a Potter-Elvehjem tissue grinder and was then shaken vigorously for 45 min at 37°C and centrifuged for 30 min at 39,600 x g at 4°C. The supernatant was discarded and the sediment resuspended and homogenized in fresh serum albumin as described above. The 45-min extraction at 37°C and the 30-40 min centrifugation at 39,600 x g were repeated five times. The sediment thus obtained was twice washed and resedimented with distilled water at 4°C and lyophilized. These preparations will be further designated as $R_{C,H,A,S}$.

The above serum albumin extraction procedure is a modification of that used by Fleischer and Fleischer (1967) for the extraction of lysophospholipids and fatty acids from beef heart mitochondria.

5.2.3. *Spectral measurements*

Absorption spectra of dry rhodopsin preparations ($R_{C,H,A}$ or $R_{C,H,A,S}$) were recorded after extraction with 1°/o Triton X-100 as described previously (2.2.7). The rhodopsin content of the extracts was calculated from the ΔA_{500} per mg preparation as described under 2.2.3.

During the phospholipase A incubation rhodopsin was determined by adding 50-μl aliquots of the incubated suspension to 400 μl of a 1^{o}/o Triton X-100 solution in 67 mM phosphate buffer (pH 6.8). The mixture was then homogenized in a Potter-Elvehjem tissue grinder. It was allowed to stand for 10 min, was again homogenized and centrifuged for 5 min at 18,100 x g. From the ΔA_{500} of the supernatant solution (2.2.3) the rhodopsin content was calculated.

In order to determine the effect of the bovine serum albumin extraction on the rhodopsin content, an amount of $R_{C,H,A}$ was incubated at 37^{o}C in a 1^{o}/o albumin solution in glycylglycine buffer of pH 7.4 (2 mg $R_{C,H,A}$/ ml buffer) for up to five hours. At certain intervals 75- μl aliquots were taken and added to 400 μl of a 1^{o}/o Triton X-100 solution in 67 mM phosphate buffer (pH 6.8). The mixture was homogenized in a Potter-Elvehjem tissue grinder, allowed to stand for 10 min, then again homogenized and centrifuged for 5 min at 18,100 x g. From the ΔA_{500} of the supernatant solution (see 2.2.3) the rhodopsin content was calculated.

5.2.4. *Lipid extraction*

For complete lipid extraction $R_{C,H,A}$ and $R_{C,H,A,S}$ were extracted three times for one hour with 0.1 ml of a $CHCl_3$ - CH_3OH - H_2O mixture (60 : 30 : 3.6, by vol.) per mg preparation as described under 3.2.2. In order to prevent loss of lysophosphatidyl serine into the aqueous phase the total lipid extracts were not washed with 0.1 M KCl prior to analysis.

5.2.5. *Phospholipid analysis*

The phospholipids present in $R_{C,H,A}$ and $R_{C,H,A,S,}$ were analyzed qualitatively as well as quantitatively by two- dimensional thin-layer chromatography as described in 3.2.4 and 3.2.6.

5.3.1. *Enzymatic hydrolysis of phosphatidyl serine*

Since phospholipase C treatment did not decrease the level of phosphatidyl serine in rod outer segments to a value below 1.6 mole per mole of chromophoric retinaldehyde, several methods were tried to bring this value to a lower level.

The phospholipase C treated-hexane extracted preparations were reincubated with phospholipase C (1.5 hr, 37°C), in the hope that the removal of the diglycerides by the hexane extraction following the first phospholipase C incubation would render the residual phosphatidyl serine more accessible to hydrolysis by a second phospholipase C treatment. When the lipid extracts of the preparations treated in this way were subjected to two-dimensional thin-layer chromatography, it appeared that the residual phosphatidyl serine had not been hydrolyzed to a significant degree. This lack of hydrolysis might be due to the fact that the phosphatidyl serine is protected by the sterical structure of the protein to which it is attached. Therefore, the phospholipase C treated-hexane extracted preparations were treated with 6 M urea for 30 min at pH 6.2 according to the method of Kito and Takezaki (1966). Under these conditions the spectral integrity of native rhodopsin is hardly or not at all attacked (Kito and Takezaki, 1966). The resulting preparations were washed twice with distilled water and incubated with phospholipase C for one hour at 37°. This method did not cause further removal of phosphatidyl serine either, as shown after lipid analysis by thin-layer chromatography. Moreover, during this treatment about 70°/o of the rhodopsin present in the phospholipase C treated-hexane extracted preparations was lost as indicated by the decrease in ΔA_{500}.

Treatment with phospholipase A_2 appeared to be more successful. As shown in Fig.17, phospholipase A_2 hydrolyses a phospholipid to the corresponding lysophospholipid by removal of a fatty acid. When phospholipase C treated-hexane extracted preparations were incubated for 1.5 hours at 37° with snake venom from Crotalus adamanteus, it appeared from qualitative chromatographic analysis of the lipid extract of these preparations that most of the phosphatidyl serine was converted into lysophosphatidyl serine.

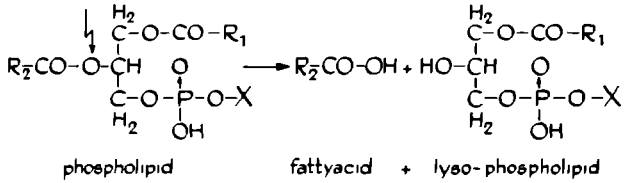


Figure 17. Mode of action of phospholipase A_2.
X represents the polar end-group of the phospholipid.

In order to determine the effect of this treatment on the rhodopsin content, aliquots were taken from the incubation suspension at various intervals for determination of the amount of intact rhodopsin present. Fig.18

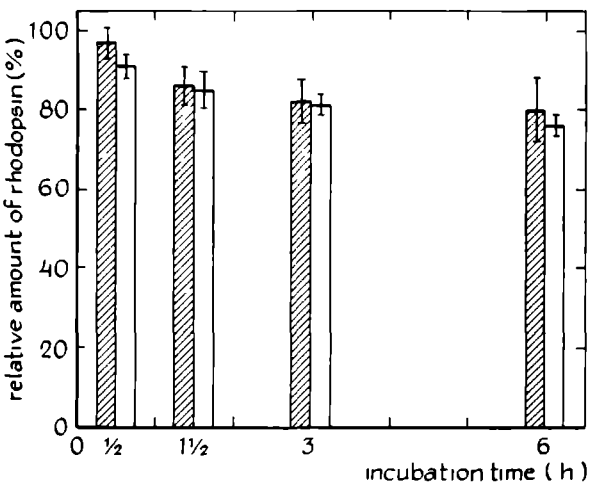


Figure 18. Effect of incubation with (shaded bars) and without (white bars) added phospholipase A_2 on the rhodopsin content.
Phospolipase C treated-hexane extracted rod preparations were incubated for 1.5 hours at pH 7.2 and 37°C. The lengths of the bars represent the means (with standard errors) of four determinations each. The amount of rhodopsin present at the start of the incubation was taken as 100°/o.

indicates that the spectral integrity of the rhodopsin was only slowly affected by incubation with the snake venom. After 1.5 hours of incubation the amount of spectrally intact rhodopsin had decreased to 86°/o (S.E. 4.8°/o, n = 4) of that present at the start of the incubation. Simultaneous incubation of the same mixture without phospholipase A gave the same decrease in rhodopsin content (Fig.18). The shape of the absorption spectrum of rhodopsin in $R_{C,H,A}$ is shown in Fig.19. It does not differ significantly from that of the rhodopsin present in phospholipase C treated- hexane extracted prepara-

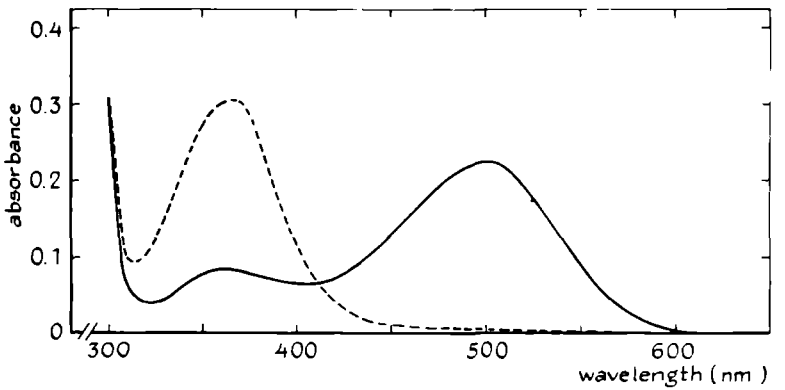


Figure 19. Absorption spectra of phospholipase C treated-hexane extracted, phospholipase A treated rod preparations before (—) and after (.....) illumination. The spectra were measured in a solution of 1°/o Triton X-100 in 67 mM phosphate buffer pH 6.8 to which hydroxylamine was added to a final concentration of 48 mM.

tions (see Fig.14), with the exception of the absorption peak at 360 nm which appears to be more pronounced. This may be due to the presence of some retinaldehyde originating from rhodopsin decomposed during incubation.

5.3.2. *Extraction of lysophosphatidyl serine*

In order to decrease the molar ratio of lipid serine to retinaldehyde to a minimal level it was necessary to remove the lysophosphatidyl serine still

present in the $R_{C,H,A}$ preparations. For this purpose we extracted the $R_{C,H,A}$ preparations with a solution of serum albumin.

The effect of this treatment on the rhodopsin content of the preparation was determined by taking aliquots at various intervals during a 5-hr incubation of $R_{C,H,A}$ in serum albumin solution at 37°C. Fig.20 shows that after this period the amount of rhodopsin had decreased to 74°/o (S.E. 7.7°/o; n = 3) of the amount present at the start of the incubation. This means that the $R_{C,H,A,S}$ preparation contains about 64°/o of the amount of rhodopsin present in the original native rod outer segment preparation. Fig.20 also demonstrates that the decrease in the amount of rhodopsin is the same when serum albumin is omitted from the extraction medium.

The absorption spectrum before and after illumination of the rhodopsin present in $R_{C,H,A,S}$ is represented in Fig.21. It appears that the shape of the

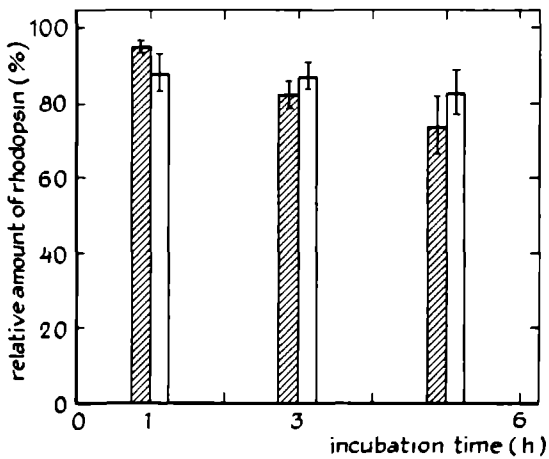


Figure 20. Effect of serum albumin treatment on rhodopsin content of phospholipase C treated-hexane extracted, phosholipase A treated rod preparations.
Shaded bars represent treatment with 1°/o serum albumin in glycylglycine buffer. White bars represent treatment with glycylglycine buffer without added serum albumin. The lengths of the bars represent the means (with standard errors) of three determinations each. The initial amount of rhodopsin was taken as 100°/o.

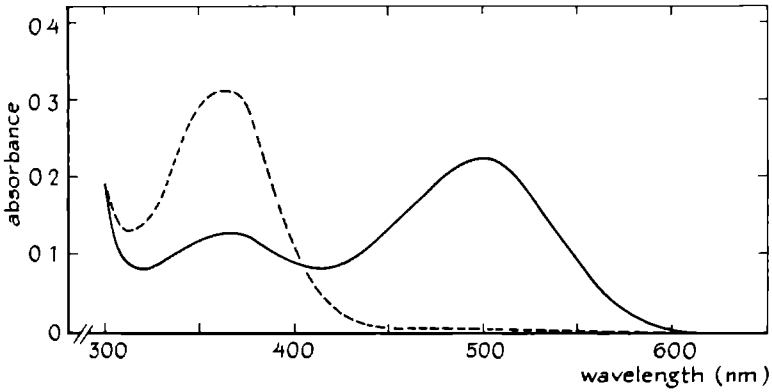


Figure 21 Absorption spectra of a phospholipase C treated-hexane extracted, phospholipase A treated, serum albumin treated rod preparation before (——) and after illumination ( )
The spectra were measured in a solution of 1% Triton X 100 in 67 mM phosphate buffer pH 6 8 to which hydroxylamine was added to a final concentration of 48 mM

two spectra are practically the same as those of the $R_{C,H,A}$ preparations (Fig.19)

The results presented in Table IX demonstrate that the $R_{C,H,A,S}$ preparations contain only 0 56 μg lipid-P per mg From this value and the amount of spectrally intact rhodopsin present per mg preparation (determined from the ΔA_{500} per mg), we calculated that only 2 7 moles of lipid-P were present per mole of chromophoric retinaldehyde. A quantitative analysis of these phospholipids by means of two-dimensional thin-layer chromotography on silicagel showed (Table X) that the major compounds were sphingomyelin and diphosphatidyl glycerol, whereas phosphatidyl serine, which is a prominent component of the phospholipase C treated-hexane extracted preparations (Table VII), was only present in small quantities Lysophosphatidyl serine was completely absent in the $R_{C,H,A,S}$ preparation which indicated that it was extracted very effectively by the serum albumin treatment From the data in

TABLE IX

LIPID-PHOSPHORUS CONTENT OF ROD OUTER SEGMENT PREPARATIONS AFTER VARIOUS TREATMENTS

Values are given with their standard errors and in parentheses the number of preparations analyzed

Rod outer segment preparation used	μg Lipid P / mg rod preparation	Lipid-P * / retinaldehyde
Native	12 6	87
Phospholipase C treated-hexane extracted	1 17 ± 0 06 (5)	4 4 ± 0 34 (4)
Ibid , phospholipase A treated-albumin extracted	0 56 ± 0 03 (4)	2 7 ± 0 17 (4)

* Molar ratio, the molar retinaldehyde content was calculated from ΔA_{500}

Table IX and X, we were able to calculate the molar ratio of the individual phospholipids to chromophoric retinaldehyde For the amino group containing phospholipids phosphatidyl ethanolamine and phosphatidyl serine ratios definitely below one were found 0 10 (S E 0 05, n = 4) and 0 10 (S E 0 01, n = 4), respectively In Fig 22 the molar content for a number of phospholipids per mole of intact rhodopsin in the $R_{C,H,A,S}$ preparations is compared with their content in native and phospholipase C treated-hexane extracted rhodopsin preparations This figure shows clearly that phospholipase A acts preferentially on the phosphatidyl serine present in the latter preparations. The molar ratios of phosphatidyl choline, sphingomyelin and diphosphatidyl glycerol to chromophoric retinaldehyde were 0 2, 0 8 and 0 2 respectively in $R_{C,H,A,S}$ They remained relatively constant during the phospholipase A and serum albumin treatments

TABLE X

PHOSPHOLIPID COMPOSITION OF RHODOPSIN AFTER TREATMENT WITH PHOSPHOLIPASE C, HEXANE, PHOSPHOLIPASE A AND ALBUMIN

The values with standard errors are averages for four preparations.

	Percent of total lipid-P
Phosphatidyl serine	3.7 ± 0.6
Lysophosphatidyl serine	not detectable
Sphingomyelin	30.0 ± 1.4
Diphosphatidyl glycerol	18.7 ± 1.7
Phosphatidyl choline	9.0 ± 0.8
Phosphatidyl inositol	4.6 ± 1.1
Phosphatidyl ethanolamine	3.9 ± 2.0
Lysophosphatidyl ethanolamine	0.4 ± 0.4
Lysophosphatidyl choline	4.0 ± 2.5
Phosphatidic acid	0.6 ± 0.5
Neutral lipids at solvent front	3.5 ± 0.9
Others	8.6 ± 2.3
Recovery	87.0 ± 6.4

5.4. DISCUSSION

In the previous chapter it could be shown that phospholipase C treatment reduced the phosphatidyl ethanolamine content significantly below 1 mole per mole chromophoric retinaldehyde without significant loss of rhodopsin, thus ruling out a link between retinaldehyde and phosphatidyl ethanolamine in native rhodopsin. In the present chapter we demonstrated that the molar ratio for phosphatidyl serine, which after phospholipase C treatment was still 1.6, could, by treatment with phospholipase A and extraction with albumin, be reduced to 0.10 based on rhodopsin present after

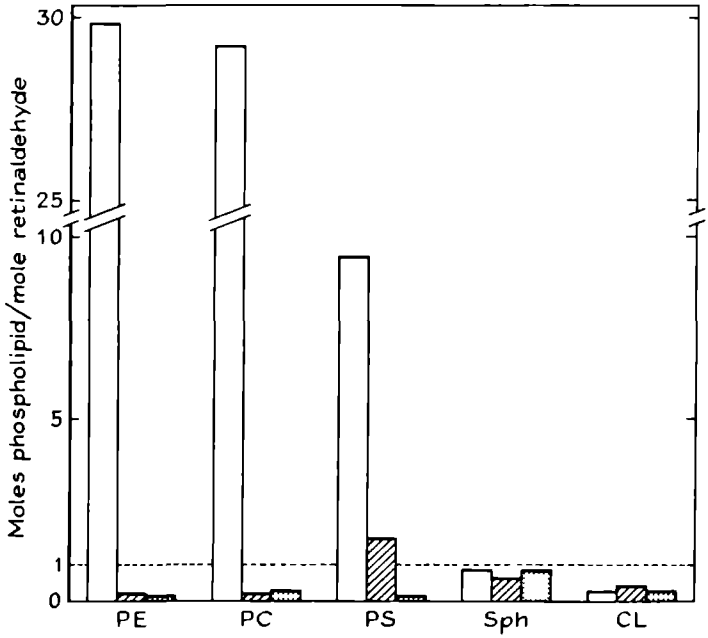


Figure 22. Phospholipid contents in native (white bars), phospholipase C treated-hexane extracted (hatched bars) and phospholipase C treated-hexane extracted, phospholipase A treated, serum albumin extracted (dotted bars) rod preparations. PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PS = phosphatidyl serine, Sph = sphingomyelin, CL = cardiolipin (diphosphatidyl glycerol).

treatment. Before drawing the conclusion that the chromophoric retinaldehyde thus can not be linked to phosphatidyl serine, it is desirable to consider the effects of these treatments on the integrity of the rhodopsin preparation.

Comparison of the findings in chapter 4 and this chapter indicates that the phospholipase C treated-hexane extracted rhodopsin preparation is less stable than native rhodopsin. Treatment namely of phospholipase C treated-hexane extracted preparations with 6M urea followed by phospholipase C treatment under conditions, which cause no loss of rhodopsin from the native preparation, destroyed 70°/o of the rhodopsin present in the phospholipase C treated-hexane extracted preparations. Similarly, incubation of a native rho-

dopsin preparation at pH 7.2 for 4 hr at 37° does not markedly reduce the rhodopsin content (Fig.13). However, incubation of phospholipase C treated-hexane extracted rhodopsin for only 1.5 hr at the same pH and temperature causes a loss of 14°/o in the rhodopsin content (Fig.18).

Incubation during the same time in the presence of phospholipase A, giving the $R_{C,H,A}$ preparation, resulted in the same loss of rhodopsin. The effect of the bovine serum albumin treatment on the integrity of rhodopsin was determined by incubating the $R_{C,H,A}$ preparation with serum albumin for up to five hours without refreshing the extractant. A single 5-hr extraction, rather than five extractions for one hour each with fresh albumin solutions, was chosen in order to avoid loss of rod outer segment material due to the frequent changing of the albumin containing medium. In this single 5-hr extraction period a loss of 26°/o of the rhodopsin present in $R_{C,H,A}$ was found (Fig.20). Omission of the serum albumin from the incubation medium gave the same loss of rhodopsin. Hence, this loss was due to keeping the pigment preparation for 5 hr in aqueous suspension at pH 7.2. Consequently, we may expect that an equal loss of rhodopsin occurred during the five 1-hr extraction periods with serum albumin used for the preparation of $R_{C,H,A,S}$. This means that incubation with phospholipase A followed by the repeated extractions with serum albumin cause a loss of 36°/o of the rhodopsin present in the native rod preparations. Such a loss is not large enough to invalidate the conclusion that the ratio of 0.10 mole phosphatidyl serine per mole of intact rhodopsin present in $R_{C,H,A,S}$ rules out a link between chromophoric retinaldehyde and phosphatidyl serine in this preparation.

Before we can reach the same conclusion for phosphatidyl serine in native rhodopsin from the values in Fig.22, we must consider the possibility that during phospholipase A and albumin treatment of the rhodopsin there might occur a transiminization of retinaldehyde from the amino group of phosphatidyl serine to another amino group in opsin. However, the shape of the absorption spectrum of rhodopsin remains practically unchanged during the various treatments (Figs.19 and 21). Since it is highly unlikely that there would exist two rhodopsins with different binding sites for the retinaldehyde but with identical spectral properties, we conclude that in native cattle rhodopsin the retinaldehyde cannot be linked to either phosphatidyl ethanolamine (see Chapter 4) or to phosphatidyl serine.

The same conclusion has been reached by Daemen et al (1971), who

applied an entirely different approach They extracted native rod outer segment preparations with neutral or acidified methanol. In parallel experiments a synthetic retinylidene lysine aldimine with a 25-fold molar excess of phosphatidyl ethanolamine was treated with neutral or acidified methanol, whereupon the aldimine mixture was analyzed under conditions excluding transiminisation. It was found that N-retinylidene phosphatidyl ethanolamine could only be extracted from rhodopsin by methanol containing 0 - 10^{-3} M HCl, under which conditions transiminisation of retinaldehyde from lysine to phosphatidyl ethanolamine was observed in the parallel experiments. At higher acid levels no chromophore was extracted from rhodopsin, while in the parallel experiments no transiminisation occured. Their conclusion is that retinaldehyde is not bound in native rhodopsin through an aldimine link to a phospholipid, but is probably bound to the ϵ-amino group of a lysine residue in opsin.

Another point to be considered here is the yield of $R_{C,H,A,S}$ relative to the amount of phospholipase C treated-hexane extracted rod preparation used for the phospholipase A-serum albumin treatment. It is difficult to determine this yield with reasonable accuracy, just by weighing, since the large number of centrifugation steps necessary in the repeated albumin procedure may easily cause unreproducible losses of $R_{C,H,A}$ material. There is, however, another method to achieve a rough estimate of this yield. We showed that 64°/o of the amount of rhodopsin present after treatment with phospholipase C and hexane extraction remains intact upon treatment with phospholipase A and serum albumin. Simultaneous determination of the ΔA_{500} per mg of the $R_{C,H,A,S}$ preparation gave a value of 102°/o relative to that of the phospholipase C treated-hexane extracted preparation. Assuming that no substantial amount of rhodopsin is solubilized during the phospholipase A treatment and subsequent albumin extraction, then this means that 37°/o non-rhodopsin material is removed on a weight basis from the phospholipase C treated-hexane extracted preparations by this procedure. The experimentally observed weight loss was larger (60°/o), presumably due to loss of extraneous material inherent in the procedure. Since the phospholipase C treated-hexane extracted preparations contain only 3°/o phospholipids by weight, the extracted material must consist largely of non-lipid material, probably protein other than rhodopsin.

5.5. SUMMARY

Phospholipase C treated-hexane extracted rod preparations were incubated with phospholipase A_2 from Crotalus adamanteus. Qualitative lipid analysis indicated that most of the phosphatidyl serine was then hydrolyzed to lysophosphatidyl serine. During this treatment 86°/o of the amount of rhodopsin originally present remained spectrally intact.

In order to remove the lyso compound, the phospholipase A treated preparations were extracted with an aqueous solution of bovine serum albumin. Quantitative analysis of the phospholipids present in the albumin extracted preparations showed that lysophosphatidyl serine had been completely removed by the serum albumin. The molar ratio of phosphatidyl serine to chromophoric retinaldehyde was reduced from 1.6 before phospholipase A treatment to 0.10 after serum albumin extraction. After the albumin treatment the amount of spectrally intact rhodopsin was decreased to 64°/o of that present in the native rod preparations. The molar ratio between phosphatidyl ethanolamine and chromophoric retinaldehyde had a value of 0.10 in the albumin extracted preparations. We conclude from these findings that retinaldehyde can neither be linked to phosphatidyl serine nor to phosphatidyl ethanolamine in native rhodopsin.

CHAPTER 6

THE REGENERATING CAPACITY OF RHODOPSIN

6.1. INTRODUCTION

In the chapters 4 and 5 it has been shown that removal of by far the largest part of the phospholipids present in native rhodopsin preparations affects the spectral integrity of the visual pigment very little. The question arises whether this extensive removal of phospholipids affects other properties of the rhodopsin. The most essential property of rhodopsin, besides its photolytic capability, is its regenerating capacity. The latter can be defined as the ability of opsin, i.e. bleached rhodopsin, to form rhodopsin upon incubation with added 11-cis retinaldehyde in the dark (Hubbard and Wald, 1952).

It was observed that upon ageing of rhodopsin preparations the regenerating capacity of rhodopsin decreased, while on the other hand the absorbance at 500 nm before photolysis remained unaltered (Radding and Wald, 1956 b). Treatment of rhodopsin with sulfhydryl reagents like p-chloromercuribenzoate also decrease the regenerating capacity without influencing the absorption spectrum of the unbleached rhodopsin (Wald and Brown, 1951). These observations indicate that the spectral integrity and the regenerating capacity are two measures for the intactness of a rhodopsin preparation which are rather independent from each other.

In this chapter we describe preliminary results of a study of the regenerating capacities of rhodopsin before and after treatment with phospholipase C, hexane, phospholipase A and serum albumin. From the results obtained thus far, it appears that the phospholipase C treatment causes a large decrease in the regenerating capacity of rhodopsin. The subsequent treatments with hexane, phospholipase A and serum albumin appear to have no significant effect. The absence of an effect of phospholipase C treatment on the spectral and photolytic properties of rhodopsin suggests that this treatment causes a structural change in the molecule while leaving the environment of the chromophoric binding site unaffected.

6.2. MATERIALS AND METHODS

6.2.1. *Preparation of the various rod outer segment preparations*

Native rod outer segment preparations were prepared as described in 2.2.1. Phospholipase C treatment, hexane extraction, phospholipase A treatment and serum albumin extraction were carried out as described in 4.2.3., 4.2.4., 5.2.1. and 5.2.2. respectively.

6.2.2. *Determination of the regenerating capacity*

A quantity of dry rod outer segment preparation was homogenized in a Potter-Elvehjem tissue grinder in 0.067 M phosphate buffer (pH 6.3) containing 0.36 M sucrose (4-6 mg rod preparation/ml buffer). The amount of rhodopsin present was determined in an aliquot of the suspension by mixing this sample with a 1°/o Triton X-100 solution in 67 mM phosphate buffer pH 6.8 (100 µl suspension/300 µl Triton X-100 solution) and measuring the absorbance at 500 mm before and after illumination in the presence of hydroxylamine as described in 4.2.7.

The remaining suspension was illuminated by a 75 W tungsten lamp at a distance of 20 cm for 20 min through infrared and orange filters (GG-3 and OG-2 filters, thickness 3mm each, Schott-Jena, Mainz, Germany). During this period the suspension was shaken at 5-min intervals. Completeness of the photolysis was checked by mixing an aliquot of the illuminated suspension with 1°/o Triton X-100 (100 µl suspension/300 µl Triton X-100 solution) and determining the amount of rhodopsin in the presence of hydroxylamine as described in 4.2.7.

To the then remaining suspension a solution of 11-cis retinaldehyde (prepared as described in 2.2.2.) in acetone was added. Unless indicated otherwise, 3-6 moles of 11-cis retinaldehyde were added per mole of rhodopsin present before illumination. The volume of acetone in which the 11-cis retinaldehyde was dissolved never exceeded 10 µl per 200 µl rhodopsin suspension. The mixture was generally incubated for 1.5 hr under vigorous shaking in the dark in a nitrogen atmosphere. At the end of the incubation period an aliquot of the suspension was mixed with 3 volumes 1°/o Triton X-100. The amount

of regenerated rhodopsin was determined from the decrease in the absorbance at 500 nm in the presence of hydroxylamine upon illumination of the Triton extract (see 4.2.7.).

6.3. RESULTS

When the regenerating capacity of rhodopsin is to be determined, the ΔA_{500} of the pigment preparation must first be measured. The preparation is then illuminated in the absence of hydroxylamine and subsequently incubated in the dark with 11-cis retinaldehyde in order to achieve regeneration of the rhodopsin. The amount of regenerated rhodopsin is then determined by measuring the decrease in the absorbance at 500 nm upon illumination in the presence of hydroxylamine. The amount of rhodopsin thus found is expressed in percent of the amount of rhodopsin originally present in the preparation. This precentage is designated by us as the regenerating capacity of the rhodopsin.

Fig.23 shows the spectrum of the product obtained by incubation of an illuminated native rod preparation with 11-cis retinaldehyde. The spectrum of the non-illuminated product in the presence of hydroxylamine shows a peak at 500 nm characteristic for rhodopsin and a large peak at 360 nm. The latter peak is due to the presence of an excess of 11-cis retinaldehyde which has been converted by hydroxylamine into its oxime. Illumination of the preparation causes photolysis of the regenerated product, resulting in the disappearance of the 500 nm peak as well as in an enlargement of the absorbance at 360 nm due to the liberation of retinaldehyde. These observations indicate that the product formed upon incubation with 11-cis retinaldehyde is indeed rhodopsin.

Since we wanted to determine the influence of phospholipase C treatment, hexane extraction, phospholipase A treatment and serum albumin extraction on the regenerating capacity of rhodopsin, we compared the regenerating capacity obtained after these various treatments with that of the native rod preparations.

Fig.24 illustrates the regeneration of rhodopsin in the various preparations as a function of the time of incubation with 11-cis retinaldehyde. From the shape of the curves it can be seen that maximal or nearly maximal regeneration is achieved after 1.5 hr of incubation with 11-cis retinaldehyde.

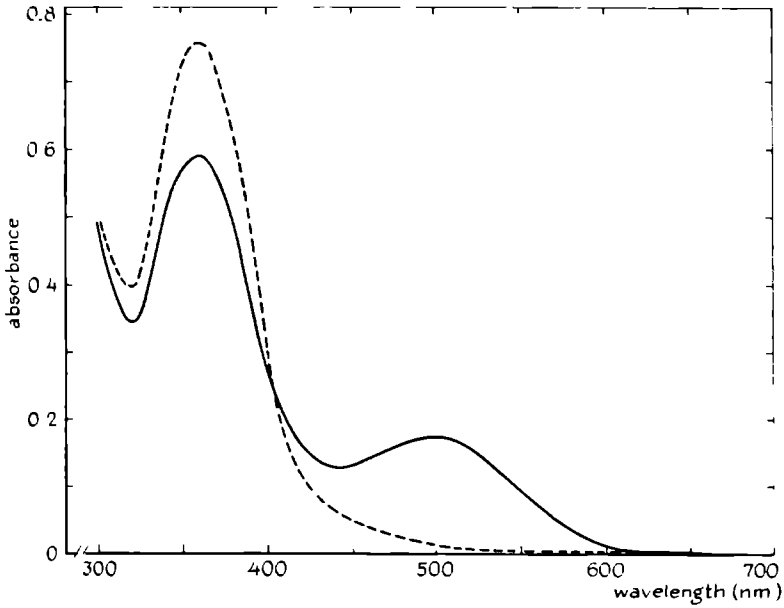


Figure 23. Spectra of regenerated rhodopsin before (—) and after (- - - -) illumination. The spectra were measured in 0.25% aqueous Triton X-100 in the presence of hydroxylamine (0.48 mM). The regeneration product was obtained by incubation of illuminated native rod preparations with excess 11-cis retinaldehyde in the dark.

Table XI shows the regenerating capacities of rhodopsin in the native and treated preparations as found after 1.5 hr of incubation with 11-cis retinaldehyde. The values illustrate that the regenerating capacity of rhodopsin is dramatically affected by phospholipase C incubation, and that it is not further altered by subsequent hexane extraction and phospholipase A treatment. Increasing the amount of added 11-cis retinaldehyde to 24 moles per mole of rhodopsin did not enhance the regenerating capacity of the phospholipase C treated preparations. The slightly higher regenerating capacity after serum albumin treatment was not significantly different (Wilcoxon test: $P_{bil.} = 0.12$; Wilcoxon, 1945) from that observed before these treatments.

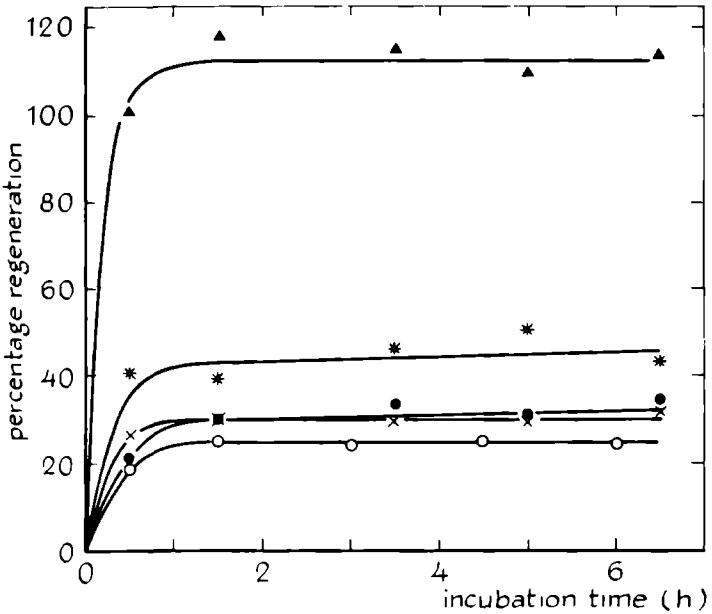


Figure 24. Regenerating capacity of rhodopsin after various treatments.
Amounts of regenerated rhodopsin in native (▲——▲), phospolipase C treated (x——x), phospholipase C treated - hexane extracted (●——●), ibid - phospholipase A treated (o——o), ibid - phospholipase A treated - serum albumin extracted (*——*) rod preparations upon incubation of the illuminated preparations with 11-cis retinaldehyde are shown. The amount of rhodopsin present in the non-illuminated preparations was always taken as 100°/o.

A further comment with regard to the values listed in tabel XI is in order. Illumination of the preparations in the absence of hydroxylamine prior to incubation with 11-cis retinaldehyde does not cause complete photolysis of rhodopsin. In order to determine the amount of residual rhodopsin, aliquots of the suspensions illuminated in the absence of hydroxylamine were used for a determination of ΔA_{500} in Triton X-100 containing 48 mM hydroxylamine. This ΔA_{500} was expressed in percent of the ΔA_{500} of the original preparation before illumination. The values thus found for the various preparations are given in table XI, column 2. Further illumination did not lower

TABLE XI

REGENERATING CAPACITY OF RHODOPSIN AFTER VARIOUS TREATMENTS

Values are given with their standard errors, numbers between parentheses represent the number of rod preparations investigated The amount of rhodopsin present in the non-illuminated preparations before incubation with 11-cis retinaldehyde was taken as 100%

Rod preparation	Percentage * regeneration	Not photolyzed **
Native	113 ± 4 2 (9)	14 ± 3.4 (7)
Pl-ase C treated	30 ± 4.1 (13)	15 ± 1.7 (8)
Pl-ase C treated-hexane extracted	30 ± 4 5 (9)	19 ± 4.0 (5)
Ibid, Pl-ase A treated	25 ± 1 8 (3)	17 ± 3 8 (3)
Ibid, Pl-ase A treated-albumin extracted	42 ± 5.3 (3)	18 ± 3.5 (3)

* The regenerating capacities were measured after 1 5 hr of incubation with 11-cis retinaldehyde

** Represents the residual amount of rhodopsin found in preparations illuminated in the absence of hydroxylamine prior to incubation with 11-cis retinaldehyde

these values. The relationship between the latter values and the regenerating capacity of rhodopsin will be discussed in section 6 4.

Next the question arose whether the action of phospholipase C or merely the incubation causes the large decrease in regenerating capacity during phospholipase C incubation. This matter was investigated by performing con-

trol incubations, in which addition of phospholipase C to the incubation medium was omitted The values in the second column of table XII indicate that two of the four preparations lost a considerable part of their regenerating capacity upon incubation without phospholipase C However, all four preparations showed a considerably lower regenerating capacity when incubated in the presence of phospholipase C Application of the Student t-test for paired observations showed this effect to be statistically significant ($t = 2\ 92$, $P = 0\ 03$, one sidedly) Thus, it seems that phospholipase C has an adverse effect on the regenerating capacity of rhodopsin, but that the incubation conditions can also adversely affect this property in certain preparations

TABLE XII

INFLUENCE OF INCUBATION WITH OR WITHOUT ADDED PHOSPHOLIPASE C ON THE REGENERATING CAPACITY OF RHODOPSIN

	Percentage regeneration *	
Rod preparation	Pl-ase C incubation	Incubation without added Pl-ase C
I	35	79
	28	80
II	26	105
	24	97
III	19	34
	6	25
IV	29	46
	30	53

* The amount of rhodopsin present in the unilluminated preparations was taken as 100%

The first point deserving attention is the incomplete photolysis observed upon illumination of aqueous rhodopsin suspensions in the absence of added hydroxylamine (Table XI, column 2). This observation can be explained in two ways. First, part of the rhodopsin molecules in the preparations would not be reached by light, resulting in an incomplete photolysis of the visual pigment. Secondly, the rhodopsin would be photolyzed completely, but during photolysis a simultaneous, partial regeneration of rhodopsin occurs. The first explanation appears unlikely, because the fragments in the suspensions were very finely divided by prior homogenization and in addition the suspensions were periodically shaken during illumination, while doubling the time of illumination had no effect. The second explanation would involve partial regeneration of rhodopsin caused either by photoregeneration of one or more photo-products of rhodopsin (Matthews et al, 1963) or by photoisomerisation of free all-trans retinaldehyde to 11-cis retinaldehyde (Hubbard and Wald, 1952), which then combines with opsin under formation of rhodopsin. Photoregeneration from one of the photoproducts of rhodopsin seems unlikely. The lifetime of these products at room temperature is too short to yield significant photoregeneration at the relatively low light intensities used in our experiments (Williams, 1968). Photoisomerisation of free retinaldehyde to the all-trans isomer is also unlikely to occur in our experiments, because illumination of the preparations before incubation with 11-cis retinaldehyde was carried out with orange light of a wavelength larger than 550 nm. Absorption of light of these wavelengths by free retinaldehyde is negligible and hence also its photoisomerization.

Hence neither one of the two possible explanations for the partial bleaching of the rhodopsin seems satisfactory. This means that it is not clear whether or not the percentages of regeneration (Table XI, column 1) should be corrected for the values in column 2. This implies that we cannot say whether the regenerating capacity of the phospholipase C incubated preparations is $30^{o}/o$ or $15^{o}/o$. However, even the lower figure still represents a significant regeneration (Student t-test $t = 3.4$, $P = 0.003$).

Table XI shows that the amount of regenerated rhodopsin in the native rod preparations (113 ± 4.2; 9 detns) significantly exceeds the amount of rhodopsin originally present. The most obvious explanation for this phenom-

enon would be that some opsin is present in the rod outer segment preparations. Formation of opsin during the isolation procedure of the outer segments is not likely, since all steps have been carried out in dim red light ($\lambda > 620$ nm) or in complete darkness. More likely, this is due to the fact that the animals were slaughtered under conditions of illumination, where part of the rhodopsin is in the bleached state and fails to regenerate during the 1-2 hr in which the isolated eyes were kept in darkness at room temperature before removal of the retina. This explanation has been further substantiated by de Grip in our laboratory (W.J. de Grip, unpublished observations), who has been able to develop a relatively simple technique for the complete conversion of the opsin to rhodopsin in native visual pigment preparations.

As shown in table XI, the regenerating capacity of rhodopsin is dramatically lowered by treatment with phospholipase C, although the 500-nm peak and the photolytic capacity remain unchanged (see 4.3.2.). Similar observations were made by Radding and Wald (1956) in aged rhodopsin preparations, by Wald and Brown (1951) upon treatment of rhodopsin with the sulfhydryl reagent p-chloromercuribenzoate, and by Albrecht (1957) after acetylation of rhodopsin with acetic anhydride. The latter two reagents may by their reaction with sulfhydrylgroups and aminogroups, respectively, react with the groups engaged in hydrogen bonding, thus destroying the specific folding of the poly-peptide chains in native rhodopsin. Presumably, the structure in the neighborhood of the chromophoric binding site remains intact during these reactions, as indicated by the unchanged visible absorption spectrum of rhodopsin. Similarly, phospholipase C incubation might cause structural changes in the rhodopsin complex without affecting the direct environment of the chromophore.

The adverse effect of phospholipase C on the regenerating capacity of rhodopsin may be explained in various ways. First, the possibility exists that the presence of intact phospholipids is required for regeneration, the hydrolytic effect of phospholipase C on the phospholipids being directly responsible for the decrease in regeneration. Secondly, the hydrolysis of the phospholipids might make the rhodopsin complex more vulnerable to the effect of incubation. A third possible explanation would be that the phospholipase C preparation, which is a crude enzyme preparation, might contain an other enzyme, e.g. a proteolytic enzyme, capable of attacking the rhodopsin molecule. (Differences between the action of crude and purified phospholipase C

preparations from B. cereus on erythrocytes have been observed. While crude phospholipase C causes rapid hemolysis of intact erythrocytes (Chu, 1949), the purified phospholipase C does not exhibit any hemolytic activity (Zwaal et al, 1971)). This question might be clarified by incubation of the rod preparations with purified phospholipase C. Untill this can be done, we cannot decide which of the three above explanations is the correct one.

Assuming that the phospholipase C itself would be responsible for the loss of regenerating capacity, then our experiments indicate that removal down to 4 of the 87 phospholipid polar groups originally present per rhodopsin molecule leaves only 15-30^{o}/o of the original regenerating capacity intact. Krinsky's (1958) finding that removal down to 20 polar groups by Cl. perfringens phospholipase C left 85-90^{o}/o of the regenerating capacity intact suggests that a large part of the phospholipids are not essential. The effect on its regenerating capacity of reconstituting the delipidated rhodopsin preparation with graded amounts of phospholipids from rod outer segments is under investigation. In addition the effect of phospholipid removal on some other important activities present in rod outer segments is being studied. The retinol dehydrogenase activity (De Pont et al, 1970) appears not to be affected by phospholipase C treatment (Rotmans, unpublished observations), while the Na-K activated ATPase activity (Bonting et al, 1964) is greatly decreased (De Pont, unpublished observations). Also to be studied in this respect is adenyl cyclase (Bitensky et al, 1971). Such information will undoubtedly add to our understanding of the functioning of the rod sac membrane in the visual mechanism.

6.5. SUMMARY

The regenerating capacity of the rhodopsin present in variously treated rod outer segment preparations was determined from the amount of rhodopsin formed upon incubation in the dark of the illuminated preparations with 11-cis retinaldehyde. For native rod outer segment preparations the amount of rhodopsin thus regenerated was 113^{o}/o of the amount originally present. The regenerating capacity decreased sharply to 30^{o}/o upon incubation with phospholipase C, although the rhodopsin remained spectrally completely intact during this treatment. The regenerating capacity was not

changed significantly upon further treatment with hexane, phospholipase A and bovine serum albumin.

The effect of phospholipase C treatment on the regenerating capacity was largely due to the enzyme preparation, although the incubation as such also affected the regenerating capacity adversely in some cases. The possibility that contaminating enzyme activity, e.g. proteolytic activity, was responsible cannot be wholly excluded at this point.

The main conclusion, which can be drawn at this time, is that the phospholipase C treatment appears to cause a sufficiently large structural change in the rhodopsin molecule to reduce its regenerating capacity considerably, but without affecting the environment of the chromophoric binding site as judged from the lack of effect on the spectral and photolytic properties of the pigment.

SUMMARY

A crucial problem in the visual mechanism is how a photon absorbed by a visual pigment molecule can lead to a stimulation of the synaptic end of the visual receptor cell. A hypothesis for the mechanism of this process has been formulated by Bonting and Bangham in 1967. They suggested that light liberates the retinaldehyde from its original binding site in rhodopsin shifting it by a transiminisation reaction to another aminogroup in the rhodopsin-membrane complex. The blocking of the latter aminogroup would induce a local negative charge on the membrane leading to a sudden increase in cationic permeability. This would permit Na^+ ions to rush into and K^+ ions to leak out of the rod sac, thereby causing a photoreceptor current which could activate a cholinergic mechanism at the photoreceptor-bipolar synapse.

In order to know whether illumination of rhodopsin really causes transiminisation of retinaldehyde it is necessary to determine the binding site of retinaldehyde in native as well as in photolyzed rhodopsin. It has been established by various investigators that retinaldehyde in photolyzed rhodopsin is linked via an aldimine linkage with the ϵ-aminogroup of lysine. In native rhodopsin the situation was less well defined. Some observations have been reported in the literature indicating a close association between retinaldehyde in native rhodopsin and phospholipids. This led to the idea that in native rhodopsin retinaldehyde would be bound via an aldimine link to an aminogroup containing phospholipid. In terms of the Bonting - Bangham hypothesis this would mean that the transiminisation of retinaldehyde from a phospholipid to a lysine molecule would trigger visual excitation. On the other hand, there was also conflicting evidence of an indirect nature. The study described in this thesis has been directed primarily toward answering the question whether or not such a retinaldehyde-phospholipid aldimine link exists in native rhodopsin.

In chapter 1 a brief survey of our current knowledge of the morphological and chemical characteristics of the vertebrate visual system is given. Special attention has been devoted to the problem of the binding site of retinaldehyde in rhodopsin.

Chapter 2 deals with the molar absorption value of rhodopsin, a constant which is of crucial importance for the estimation of the molar concentration of the visual pigment. During the last few years a controversy over the

true value of this constant has existed, which led us to a critical study of its estimation. Since one mole of rhodopsin contains one mole of retinaldehyde, it was possible to calculate the molar absorbance coefficient from the absorbance at 500 nm and the retinaldehyde content of our preparations. A value of 43,000 l/mole.cm at 500 nm was found, confirming the traditional value of Wald and others and refuting the low value of 23,100 reported by Heller (1968a).

In chapter 3 a quantitative characterisation of the lipids present in cattle rod outer segments is presented. We found that the photoreceptor membranes contained 39°/o total lipid and 31°/o phospholipid on a dry weight basis. Fatty acid analysis revealed a strikingly high content of docosahexaenoic ($C_{22:6}$) acid, representing 34°/o (w/w) of the total fatty acids. Quantitative analysis of the phospholipids by means of two dimensional thin-layer chromatography indicated that the three major phospholipids in the rod outer segment are phosphatidyl ethanolamine (35°/o), phosphatidyl choline (35°/o) and phosphatidyl serine (11°/o). Half of the total amount of phospholipids was extractable with n-hexane without changing the spectral and photolytic properties of the rhodopsin. The molar ratios of phosphatidyl ethanolamine and phosphatidyl serine to retinaldehyde were reduced from 30 to 10 and from 10 to 5, respectively, by hexane extraction. These molar ratios for the amino group containing phospholipids still exceeded considerably the minimum ratio of 1 required for the existence of a phospholipid-retinaldehyde aldimine link in rhodopsin. Hence we sought ways to further reduce the phospholipid content of our preparations.

In chapter 4 we demonstrate that treatment of the rod outer segment preparations with phospholipase C from Bacillus cereus followed by hexane extraction resulted in a very effective removal of phospholipids from the rhodopsincomplex: 95°/o of the phospholipids originally present in the rod preparations were removed. The spectral and photolytic properties of rhodopsin remained completely intact during this treatment. Quantitative analysis of the residual phospholipids by means of two-dimensional thin-layer chromatography as well as by analysis on the amino acid analyzer, showed that the molar ratios of phosphatidyl serine and phosphatidyl ethanolamine to chromophoric retinaldehyde were reduced to 1.6 and 0.1, respectively. This observation permits the conclusion that an aldimine linkage between retinaldehyde and phosphatidyl ethanolamine cannot be present in rhodopsin.

Chapter 5 describes the removal of the phospholipase C resistant phosphatidyl serine from the phospholipase C treated-hexane extracted rod preparations. Phospholipase A_2 from Crotalus adamanteus hydrolyzed most of this phospholipid to lysophosphatidyl serine and free fatty acid. Lysophosphatidyl serine could be completely removed by means of repeated extraction with aqueous serum albumin solution. After phospholipase A treatment and serum albumin extraction the rhodopsin content was decreased by 36% when compared to the amount of rhodopsin present in the native rod preparations. Quantitative phospholipid analysis and rhodopsin determinations demonstrated that the molar ratio of phosphatidyl serine to chromophoric retinaldehyde was reduced from 1.6 to 0.10. The molar ratio between phosphatidyl ethanolamine and chromophoric retinaldehyde remained unchanged at 0.10. It was concluded from these findings that retinaldehyde in rhodopsin can be linked neither to phosphatidyl serine nor to phosphatidyl ethanolamine. Since no other aminogroup-containing phospholipids are present in sufficient amounts, this indicates the complete absence of an aldimine linkage between a phospholipid and retinaldehyde in rhodopsin.

The final chapter (Chapter 6) describes the determination of the regenerating capacity as an additional parameter of the phospholipid-denuded rhodopsin preparations. While illuminated native rhodopsin preparations yielded complete regeneration upon incubation with 11-cis retinaldehyde in the dark, the regenerating capacity was decreased to about one third of its original value by treatment of rod preparations with phospholipase C. The regenerating capacity of the phospholipase C treated preparations was not further influenced by subsequent treatment with hexane, phospholipase A or serum albumin. In contrast to the sharp decrease in regenerating capacity, treatment with phospholipase C left the rhodopsin spectrally and photolytically completely intact. This indicates that the latter treatment causes structural alterations in rhodopsin without affecting the immediate neighborhood of the chromophoric binding site.

SAMENVATTING

Een belangrijk probleem bij de bestudering van het visuele mechanisme is hoe de absorptie van een foton door een molecule visueel pigment kan leiden tot een stimulering van het synaptische uiteinde van de fotoreceptor cel. Bonting en Bangham formuleerden in 1967 een hypothese voor het mechanisme van dit proces. Zij suggereerden dat licht het retinaldehyde vrijmaakt van zijn oorspronkelijke bindingsplaats in rhodopsine en dat het door een transiminiseringsreaktie overgaat naar een andere aminogroep in het rhodopsine-membraan komplex. Het blokkeren van de laatst genoemde aminogroep zou een plaatselijke negatieve lading op het membraan induceren en aldus leiden tot een verhoogde kationen permeabiliteit. Dit zou dan tot gevolg hebben dat Na^+ ionen het staafjes zakje binnenstromen, terwijl K^+ ionen er tegelijkertijd uitlekken. Hierdoor zou een fotoreceptor stroom ontstaan waardoor een cholinergisch mechanisme in de fotoreceptor-bipolaire synaps geaktiveerd zou kunnen worden.

Om na te gaan of belichting van rhodopsine werkelijk een transiminisering van het retinaldehyde tot gevolg heeft, is het nodig de bindingsplaats van retinaldehyde in zowel onbelicht als in belicht rhodopsine te kennen. Uit het werk van verschillende onderzoekers is nu komen vast te staan dat het retinaldehyde in belicht rhodopsine via een aldimine binding gebonden is aan de ϵ-aminogroep van lysine. In onbelicht rhodopsine was de situatie veel minder duidelijk. Enkele waarnemingen uit de literatuur suggereerden het bestaan van een nauwe verwantschap tussen het retinaldehyde en fosfolipiden in natief rhodopsine. Dit leidde tot het idee dat in natief rhodopsine het retinaldehyde via een aldimine binding gebonden zou zijn aan een fosfolipide met een vrije aminogroep. In bewoordingen van de Bonting - Bangham hypothese zou dit betekenen dat de transiminisering van retinaldehyde van een fosfolipide naar een lysine molecule de visuele exitatie zou initieren. Aan de andere kant waren er ook een aantal gegevens die op indirekte wijze hiermee in strijd waren. De studie welke in dit proefschrift beschreven wordt is primair gericht geweest op de beantwoording van de vraag of er al of niet zulk een fosfolipide-retinaldehyde aldimine binding in natief rhodopsine bestaat.

In hoofdstuk 1 is een kort overzicht gegeven van onze huidige morfologische en chemische kennis van het visuele systeem in de vertebraten. Speciale aandacht is daarbij besteed aan het probleem van de bindingsplaats van retinaldehyde in rhodopsin.

Hoofdstuk 2 handelt over de waarde van de molaire absorptie koefficient van rhodopsine bij een golflengte van 500 nm. Deze waarde is van essentiele betekenis voor de bepaling van de molaire koncentratie van het visuele pigment Gedurende de laatste jaren heeft verschil van mening bestaan over de juiste waarde van deze konstante Dit bracht ons ertoe een kritische studie te wijden aan de bepaling van deze grootheid Daar ieder molecule rhodopsine één molecule retinaldehyde bevat, was het mogelijk de molaire absorptie koefficient te berekenen uit het retinaldehyde gehalte en de absorptie bij 500 nm van onze preparaten Aldus werd een waarde van 43 000 l/mol cm gevonden, waarmee de vroegere waarde van Wald en anderen bevestigd en de lage waarde van 23 000 gerapporteerd door Heller (1968a) weerlegd werd

In hoofdstuk 3 wordt een kwantitatieve karakterisering van de lipiden in het runderstaafjes buitensegment gegeven. Wij vonden dat de fotoreceptor membranen 39°/o totaal lipide en 31°/o fosfolipide op een drooggewichtsbasis bevatten Vetzuuranalysen gaven een opvallend hoog gehalte aan het sterk onverzadigde $C_{22\ 6}$ vetzuur te zien op gewichtsbasis 34°/o van de totale hoeveelheid aanwezig vetzuur Kwantitatieve analyse van de fosfolipiden door middel van twee-dimensionale dunne laag chromatografie toonde aan dat fosfatidyl ethanolamine (35°/o), fosfatidyl choline (35°/o) en fosfatidyl serine (11°/o) de drie belangrijkste fosfolipiden waren De helft van de totale hoeveelheid fosfolipiden bleek extraheerbaar met n-hexaan zonder dat de spektrale en fotolytische eigenschappen van het rhodopsine hierbij aangetast werden. De molaire verhoudingen tussen fosfatidyl ethanolamine resp fosfatidyl serine en retinaldehyde werden door hexaan extractie teruggebracht van 30 naar 10 resp. van 10 naar 5 De molaire verhoudingen van de aminogroep houdende fosfolipiden bleven dus, zelfs na de hexaan extractie, nog aanzienlijk boven de minimale waarde van 1 die vereist is voor het bestaan van een fosfolipide-aldimine binding in rhodopsine Daarom werd naar methoden gezocht waarmee het mogelijk zou zijn het fosfolipide gehalte in de staafjespreparaten verder te verlagen

In hoofdstuk 4 wordt aangetoond dat behandeling van de staafjes buitensegment preparaten met fosfolipase C van Bacillus cereus gevolgd door hexaan extractie een zeer effektieve verwijdering van fosfolipiden levert 95°/o van de oorspronkelijk aanwezige fosfolipiden konden aldus verwijderd worden De spektrale en fotolytische eigenschappen van het rhodopsine ble-

ven hierbij volledig behouden. Kwantitatieve analyse van de overgebleven fosfolipiden door middel van twee-dimensionale dunne laag chromatografie alsmede door analyse met de aminozuur analysator toonden aan dat de molaire verhoudingen tussen fosfatidyl serine resp. fosfatidyl ethanolamine en chromofoor retinaldehyde waren verlaagd tot 1,6 resp. 0,1. Deze waarneming leidde tot de konklusie dat een aldimine binding tussen retinaldehyde en fosfatidyl ethanolamine niet in rhodopsine aanwezig kan zijn.

Hoofdstuk 5 beschrijft de verwijdering van het fosfolipase C - resistente fosfatidylserine uit de fosfolipase behandelde-hexaan geextraheerde staafjes preparaten. Fosfolipase A_2 uit Crotalus adamanteus bleek een zeer groot deel van dit fosfatidyl serine te hydrolyseren tot lysophosphatidyl serine en vrij vetzuur. Lysofosfatidyl serine kon volledig worden verwijderd door herhaalde extraktie met een oplossing van serum albumine in water. Na de fosfolipase A en serum albumine behandelingen was het rhodopsine gehalte met 36% verlaagd ten opzichte van de hoeveelheid rhodopsine welke aanwezig was in onbehandelde staafjes preparaten. Kwantitatieve analyse van de fosfolipiden toonde aan dat de molaire verhouding tussen fosfatidyl serine en chromofoor retinaldehyde was gedaald van 1,6 naar 0,10. De molaire verhouding tussen fosfatidyl ethanolamine en chromofoor retinaldehyde bleef onveranderd 0,10. Uit deze resultaten werd gekonkludeerd dat retinaldehyde in rhodopsine noch aan fosfatidyl serine noch aan fosfatidyl ethanolamine gebonden voorkomt. Daar er geen andere aminogroep-houdende fosfolipiden in voldoende hoeveelheden aanwezig zijn, betekent dit dat er in rhodopsine in het geheel geen aldimine binding tussen het retinaldehyde en een fosfolipide aanwezig kan zijn.

Het laatste hoofdstuk (hoofdstuk 6) beschrijft de bepaling van het regenererend vermogen als een aanvullende parameter van de van lipiden ontdane rhodopsine preparaten. Terwijl onbehandelde belichte rhodopsine preparaten na incubatie met 11-cis retinaldehyde in het donker een volledige regeneratie van het rhodopsine te zien gaven, verminderde behandeling met fosfolipase C dit regenererend vermogen tot ca. een derde van zijn oorspronkelijke waarde. Het regenererend vermogen van de met fosfolipase C behandelde preparaten werd niet verder beinvloed door daarop volgende behandelingen met hexaan, fosfolipase A of serum albumine. In tegenstelling tot de scherpe daling van het regenererend vermogen, liet behandeling met fosfolipase C het rhodopsine spektraal en fotolytisch volledig intakt. Dit suggereert dat deze behandeling

strukturele veranderingen in het rhodopsine komplex veroorzaakt zonder daarbij de onmiddellijke omgeving van de bindingsplaats van het chromofoor aan te tasten.

REFERENCES

Abell, L.L., Levy, B.B., Brodie, B.B. and Kendall, F.E. (1952), J. Biol. Chem., **195**, 357.

Abrahamson, E.W. and Ostroy, S.E. (1967), Progr. Biophys. Mol. Biol., **17**, 179.

Adams, R.G. (1967), J. Lipid Res., **8**, 245.

Adams, R.G. (1969), J. Lipid Res., **10**, 473.

Akhtar, M. and Hirtenstein, M.D. (1969), Biochem. J., **115**, 607.

Akhtar, M., Blosse, P.T. and Dewhurst, P.B. (1967), Chem. Commun., 631.

Albrecht, G. (1957), Science, **125**, 70.

Anderson, R.E. (1970), Nature, **227**, 954.

Anderson, R.E. and Maude, M.B. (1970), Biochemistry, **9**, 3624.

Andrews, J.S. and Futterman, S. (1964), J. Biol. Chem., **239**, 4073.

Baddiley, J., Buchanan, J.G., Handschumacher, R.E. and Prescott, J.F. (1956), J. Chem. Soc., 2818.

Ball, S., Goodwin, T.W. and Morton, R.A. (1948), Biochem. J., **42**, 516.

Baumann, C.,. (1968), Pflügers Archiv, **300** R 100.

Bishel, M.D. and Austin, J.H. (1963), Biochim. Biophys. Acta, **70**, 598.

Bitensky, M.W., Gorman, R.E. and Miller, W.H. (1971), Proc. Natl. Ac. Sci. U.S., **68**, 561.

Blasie, J.K. and Worthington, C.R. (1969), J. Mol. Biol., **39**, 417.

Blasie, J.K., Worthington, C.R. and Dewey, M.M. (1969), J. Mol. Biol., **39**, 407.

Blaurock, A.E. and Wilkins, M.H.F. (1969), Nature, **223**, 906.

Bonting, S.L. (1969) in: "Current Topics in Bioenergetics" (D.R. Sanadi, ed.), vol. 3, p. 351, Academic Press, New York.

Bonting, S.L. and Bangham, A.D. (1967), Exptl. Eye Res., **6**, 400.

Bonting, S.L., Caravaggio, L.L. and Canady, M.R. (1964), Exptl. Eye Res., **3**, 47.

Borggreven, J.M.P.M., Daemen, F.J.M. and Bonting, S.L. (1970), Biochim. Biophys. Acta, **202**, 374.

Borggreven, J.M.P.M., Rotmans, J.P., Bonting, S.L. and Daemen, F.J.M. (1971), Arch. Biochem. Biophys., **145**, 290.

Bownds, D. (1967), Nature, **216**, 1178.

Bownds, D. (1970), Private communication.

Bownds, D. and Wald, G. (1965), Nature, **205**, 254.

Bridges, C.D.B. (1962), Vis. Res., **2**, 215.

Bridges, C.D.B. (1970a) in: "Biochemistry of the eye" (C.N. Graymore, e.d.), p.571, Academic Press, London.

Bridges, C.D. (1970 b), Nature, **227**, 1258.

Broda, E.E. (1941), Biochem. J., **35**, 960.

Broekhuyse, R.M. (1968), Biochim. Biophys. Acta, **152**, 307.

Broekhuyse, R.M. (1969), De fosfolipiden van de lens, Thesis, University of Nijmegen.

Brown, A.D. (1965), J. Mol. Biol., **12**, 491.

Chu, H.P. (1949), J. Gen. Microbiol., **2**, 255.

Collins, F.D. (1953), Nature, **171**, 469.
Collins, F.D., Love, R.M. and Morton, R.A. (1952), Biochem. J., **51**, 669.
Daemen, F.J.M. (1967), Chem. Phys. Lipids, **1**, 476.
Daemen, F.J.M. and Bonting, S.L. (1969), Biochim. Biophys. Acta, **183**, 90.
Daemen, F.J.M., Borggreven, J.M.P.M. and Bonting, S.L. (1970), Nature, **227**, 1259.
Daemen, F.J.M., Jansen, J.A.A. and Bonting, S.L. (1971), Archiv. Biochem. Biophys., **145**, 300.
Dartnall, H.J.A. (1961), Nature, **188**, 475.
Dartnall, H.J.A. (1962) in: "The eye" (H. Davson, ed.), p.427, Academic Press, New York.
Davison, P.F. (1968), Science, **161**, 906.
Dawson, R.M.C. (1960), Biochem. J., **75**, 45.
Dawson, R.M.C., Hemington, N. and Davenport, J.B. (1962), Biochem. J., **84**, 497.
De Pont, J.J.H.H.M., Daemen, F.J.M. and Bonting, S.L. (1968), Biochim. Biophys. Acta, **163**, 204.
De Pont, J.J.H.H.M., Daemen, F.J.M. and Bonting, S.L. (1970), Arch. Biochem. Biophys., **140**, 275.
De Robertis, E. and Lasansky, A. (1958), J. Biophys. Biochem. Cytol., **4**, 743.
Dewey, M.M., Davis, P.K., Kent Blasie, J. and Barr, L. (1969), J. Mol. Biol., **39**, 395.
Dittmer, J.D. and Lester, R.L. (1962), J. Lipid Res., **3**, 471.
Dittmer, J.D. and Lester, R.L. (1964), J.Lipid Res., **5**, 126.
Donner, K.O. and Reuter, T. (1967), Vis. Res., **7**, 17.
Donner, K.O. and Reuter, T. (1969), Vis. Res., **9**, 815.
Dowling, J.E. (1960), Nature, **188**, 114.
Dowling, J.E. (1967) in: "Molecular Organisation and Biological Function" (J.M. Allen, ed.) p. 186, Harper and Row, New York.
Eichberg, J. and Hess, H.H. (1967), Experientia, **23**, 993.
Ewald, A. and Kühne, W. (1878), Untersuch. Physiol. Inst. Univ. Heidelberg, **1**, 248.
Falk, G. and Fatt, P. (1966), J. Physiol., **186**, 104 p.
Fernandez-Moran, H. (1959), Science, **129**, 1284.
Fernandez-Moran, H. (1962), Circulation, **26**, 1039.
Fleischer, S. and Fleischer, B. (1967), in: "Methods in Enzymology" (R.W. Estabrook and M.E. Pullman, eds.), Vol X, p. 413, Academic Press, New York.
Fleischer, S. and Mc.Connell, D.G. (1966), Nature, **212**, 1366.
Fleischer, S., Fleischer, B. and Stoeckenius, W. (1967), J. Cell. Biol., **32**, 193.
Fleischer, S. and Rouser, G. (1965), J. Am. Oil Chem. Soc., **42**, 588.
Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957), J. Biol. Chem., **226**, 497.
Fridericia, L.S. and Holm, E. (1925), Am. J. Physiol., **73**, 63.
Futterman, S. and Saslaw, L.D. (1961), J. Biol. Chem., **236**, 1652.
Gras, W.J. and Worthington, C.R. (1969), Proc. Nat. Ac. Sci., **63**, 233.
De Haas, G.H., Bonsen, P.P.M. and Van Deenen, L.L.M. (1966), Biochim. Biophys. Acta, **116**, 114.

De Haas, G H., Van Zutphen, H , Bonsen, P P.M and Van Deenen, L L.M. (1964), Rec Trav Chim Pays-Bas, **83**, 99
Hagins, W A. (1956), Nature, **177**, 989
Hall, M O and Bacharach, A.D E (1970), Nature, **225**, 637
Hecht, S., Shlaer, S and Pirenne, M H (1942), J Gen Physiol , **25**, 819
Heller, J (1968 a), Biochemistry, **7**, 2906
Heller, J (1968 b), Biochemistry, **7**, 2914
Heller, J. (1969), Biochemistry, **8**, 675
Heller, J (1970), Nature, **225**, 636
Hirtenstein, M.D. and Akhtar, M (1970), Biochem. J , **119**, 359
Holm, E. (1929), Hospitalstidende, **72**, 139
Hubbard, R. (1954), J Gen Physiol , **37**, 381
Hubbard, R (1958 a), J Gen. Physiol , **42**, 259
Hubbard, R (1958 b), Nature, **181**, 1126
Hubbard, R. and Kropf, A. (1958), Proc Nat Ac. Sci U.S., **44**, 130.
Hubbard, R and Kropf, A. (1959 a), Ann. N Y. Acad Sci , **81**, 388
Hubbard, R. and Kropf, A (1959 b), Nature, **183**, 448
Hubbard, R. and Wald, G (1951), Proc Nat Ac. Sci U.S., **37**, 69
Hubbard, R. and Wald, G (1952), J. Gen Physiol , **36**, 269
Ishimoto, M. and Wald, G. (1946), Fed Proc , **5**, 50.
Kimbel, R L., Poincelot, R P and Abrahamson, E W. (1970), Biochemistry, **9**, 1817
Kito, Y and Takezaki, M. (1966), Ann Report Biol Works, **14**, 83.
Korn E.D (1968), J. Gen. Physiol , **52**, 257 S
Korn, E.D. (1969), Fed. Proc , **28**, 6.
Krinsky, N J (1958), Arch. Ophthalmol., **60**, 688
Lennaz, G., Sechi, A.M., Masotti, L. and Parenti-Castelli, G (1969), Biochem. Biophys. Res Commun., **34**, 392.
Liebman, P A (1962), Biophys. J., **2**, 161.
Lynch, M J , Raphael, S S., Mellor, L D , Spare, P.D , Hills, P. and Inwood, M.J H., (1964), Medical Laboratory Technology, p. 147, Saunders, Philadelphia.
Lythgoe, R.J. (1937), J. Physiol., **89**, 331
Matthews, R.G., Hubbard, R., Brown, P K. and Wald, G. (1963), J Gen Physiol., **47**, 215
Mc Connell, D.G. (1965), J. Cell Biol , **27**, 459
Mizuno, K., Kuno, Y and Ozawa, K (1966 a), Jap J Ophthalmol , **101**, 85.
Mizuno, K., Ozawa, K. and Kuno, Y. (1966 b), Exptl Eye Res , **5**, 276.
Moody, M F. and Robertson, J.D. (1960), J. Biophys Biochem Cytol , **7**, 87.
Morrison, W R. and Smith, L.M. (1964), J Lipid Res., **3**, 600.
Morton, R A. and Pitt, G A.J. (1949), Biochem. J., **45**, 304
Morton, R A and Pitt, G.A.J. (1957), Fortschr. Chem Org Naturstoffe, **14**, 224.
Nielsen, N C , Fleischer, S. and Mc.Connell, D.G. (1970), Biochim. Biophys. Acta, **211**, 10.

Nilsson, S.E.G. (1964), Nature, **202**, 509.
Nilsson, S.E.G. (1965), J. Ultrastructural Res., **12**, 207
O'Brien, J.S. (1965), Science, **147**, 1099.
Oroshnik, W.P., Brown, P.K., Hubbard, R. and Wald, G. (1956), Proc. Natl. Ac. Sci., U.S., **42**, 578.
Ostroy, S.E., Erhardt, F. and Abrahamson, E.W. (1966a), Biochim. Biophys. Acta, **112**, 265.
Ostroy, S.E., Erhardt, F. and Abrahamson, E.W. (1966b), Biochim. Biophys. Acta, **126**, 409.
Ottolenghi, A.C. (1965), Biochim. Biophys. Acta, **106**, 510.
Owens, K. (1964), Biochem. J., **100**, 354.
Pangborn, M.C. (1951), J. Biol. Chem., **188**, 47.
Penn, R.D. and Hagins, W.A. (1969), Nature, **223**, 20.
Perkin, J.C. and Love, B.B. (1963), Biochim. Biophys. Acta, **78**, 753.
Piez, K.A. and Morris, L. (1960), Anal. Biochem., 1, 187
Poincelot, R.P. and Abrahamson, E.W. (1970 a), Biochemistry, **9**, 1820.
Poincelot, R.P. and Abrahamson, E.W. (1970 b), Biochim. Biophys. Acta, **202**, 382.
Poincelot, R.P. and Zull, J.Z. (1969), Vis. Res., **9**, 647.
Poincelot, R.P., Millar, P.G., Kimbel, R.L. Jr. and Abrahamson, E.W. (1969), Nature, **221**, 256.
Poincelot, R.P., Glenn Millar, P., Kimbel, R.L. Jr., and Abrahamson, E.W. (1970), Biochemistry, **9**, 1809.
Pratt, D.C., Livingstone, R. and Grellmann, K.H. (1964), Photochem. Photobiol., **3**, 121.
Radding, C.M. and Wald, G. (1956 a), J. Gen. Physiol., **39**, 909.
Radding, C.M. and Wald, G. (1956 b), J. Gen. Physiol., **39**, 923.
Robertson, J.D. (1963), J. Cell Biol., **19**, 201.
Roelofsen, B.. (1968), Some studies on the extractability of lipids and the ATPase activity of the erythrocyte membrane, Thesis, University of Utrecht.
Schmidt, W.J. (1938), Kolloid. Z., **85**, 137.
Shichi, H. (1970), Biochemistry, **9**, 1973.
Shichi, H., Lewis, M.S., Irreverre, F. and Stone, A.L. (1969), J. Biol. Chem., **244**, 529.
Shields, J.E., Dinovo, E.C., Hendricksen, R.A., Kimbel, R.L. and Millar, P.G. (1967), Biochim. Biophys. Acta, **147**, 238.
Sillman, A.J., Ito, H. and Tomita, T. (1969), Vision Res. **9**, 1443.
Sjostrand, F.S. (1959), Ergebn. Biol., **21**, 128.
Sjostrand, F.S. (1961) in: "The structure of the eye" (G.K. Smelser, ed.), p.1, Acad. Press, New York.
Sjostrand, F.S. (1963) in: "Mechanisms of Demyelination" (A.S. Rose and C.M. Pearson, eds.), p.1, Mc. Graw Hill Book Company, New York.
Sjöstrand, F.S. (1968), in. "Ultrastructure in biological systems" (A.J. Dalton and F. Haguenau, eds.), p.151, Acad. Press, New York.
Sjöstrand, F.S. and Elfvin, L.S. (1964), J. Ultrastructure Res., **10**, 263.
Skidmore, W.D. and Entenman, C. (1962), J. Lipid Res., **3**, 471.

Slein, M.W. and Logan, G.F. (1965), J. Bacteriol., **90**, 69.
Storry, J.E. and Tuckley, B. (1967), Lipids, **2**, 501.
Tansley, K. (1931), J. Physiol., **71**, 442.
Tokuyasu, K. and Yamada, E. (1959), J. Biophys. Biochem. Cytol , **6**, 225.
Van Deenen, L L M. and De Haas, G.H. (1966), Ann Rev. Biochem., **35**, 178.
Vanderkooi, G and Green, D.E. (1970), Proc. Nat. Ac. Sci., **66**, 615
Vanderkooi, G. and Sundaralingam, M. (1970), Proc. Natl. Ac. Sci., **67**, 233.
Wald, G. (1933), Nature, **132**, 316.
Wald, G. (1935), J. Gen Physiol., **19**, 351.
Wald, G. (1956), in "Enzymes. units of biological structure and function" (O. Gaebler, ed.), p.355, Acad. Press, New York.
Wald, G. (1961), in: "The structure of the eye" (G.K. Smelser, ed.), p.421, Acad. Press, New York
Wald, G. and Brown, P.K. (1952), J. Gen. Physiol., **35**, 797.
Wald, G. and Brown, P.K. (1953), J. Gen. Physiol., **37**, 381.
Wald, G., Brown, P.K. and Gibbons, J.R. (1963), J. Opt Soc. Am., **53**, 20.
Wald, G., Durell, J. and St. George, R.C.C. (1950), Science, **111**, 179.
Weale, R.A. (1957), Brit. J. Ophthalmol., **41**, 461.
Wilcoxon, F. (1945), Biometrics (Bulletin), **1**, 80.
Williams, T.P. (1968), Vis. Res., **8**, 1457.
Wood, P.D.S. and Holton, S. (1964), Proc. Soc. Exp. Biol. Med., **115**, 990.
Yoshizawa, T. and Wald, G., (1963), Nature, **197**, 1279.
Yoshizawa, F., Kito, Y. and Ishigami, M. (1960), Biochim. Biophys. Acta, **43**, 329.
Young, R.W. and Droz, B. (1968), J. Cell. Biol., **39**, 169.
Zorn, M. and Futterman, S. (1971), J. Biol. Chem., **246**, 881.
Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1971), Biochim. Biophys Acta, **233**, 474.

STELLINGEN

I

De veronderstelling van Poincelot en medewerkers, dat retinaldehyde in rhodopsine via een aldimine binding aan fosfatidyl ethanolamine gebonden is, is onjuist.

Poincelot, R.P., Millar, P.G., Kimbel, R.L. and Abrahamson, E.W., Nature, 221 (1969) 256.
Poincelot, R.P., Millar, P.G., Kimbel, R.L. and Abrahamson, E.W., Biochemistry 9 (1970) 1809.

II

De experimenten van Mc.Donald en Korner rechtvaardigen niet hun konklusie, dat groeihormoon specifiek de eiwitsynthese aan membraangebonden ribosomen stimuleert.

Mc.Donald, R.I. and Korner, A., FEBS Letters, 13 (1971) 62.

III

De vorming van alkyl halogeniden bij de reaktie van t-butyl hypochloriet met ethers, zoals voorgesteld door Walling en Mintz, kan ook worden verklaard door ontleding van de eveneens gevormde α-chloor ethers via een ionogeen mechanisme.

Walling, C. and Mintz, M.J., J.Am. Chem. Soc., 89 (1967) 1515.

IV

De veronderstelling van Moake en medewerkers, dat de adenosinedifosfaat-geinduceerde bloedplaatjesaggregatie in oorzakelijk verband zou staan met het door adenosinedifosfaat geremde, Mg^{2+} - afhankelijke, $(Na^+ + K^+)$-gestimuleerde adenosinetrifosfatase systeem, is onwaarschijnlijk.

Moake, J.L., Ahmed, K., Bachur, N.R. and Gutfreund, D.E., Biochim. Biophys. Acta, 211 (1970) 337.

V

Bij de bestudering van de verschillen in osmotische resistentie van erythrocyten in elektrolyt- en niet-elektrolytoplossingen, dient men rekening te houden met de tijdens zwelling optredende verhoging van het passieve kationentransport door het celmembraan.

Coldman, M.F., Gent, M. and Good, W., Comp. Biochem. Physiol., 33 (1970) 157.

VI

De argumenten welke Zorn en Futterman aanvoeren voor hun veronderstelling, dat fosfolipiden essentieel zijn voor de spektrale intaktheid van rhodopsine, zijn niet doorslaggevend.

Zorn, M. and Futterman, S., J. Biol. Chem., 246 (1971) 881.

VII

Het is onwaarschijnlijk dat de temperatuuraktivering van F_1-adenosinetrifosfatase uit runderhartmitochondriën, door dissociatie van een inhibitor wordt veroorzaakt.

Horstman, L.L. and Racker, E., J. Biol. Chem. 245 (1970) 1336.

VIII

De konklusie van Alcock en Shills, dat een neutrale pyrofosfatase een rol zou spelen bij de verkalking in kraakbeen, berust op onvoldoende experimentele gegevens.

Alcock, N.W. and Shills, M.E., Biochem. J., 112 (1969) 505.

IX

De oprichting van een uitgebreid netwerk van proefstanden voor de kontrole van de geluidssterkte geproduceerd door gemotoriseerde voertuigen, verdient uit milieuhygienisch oogpunt sterke aanbeveling.

J.M.P.M. Borggreven
8 oktober 1971.